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jc584 U.S. PTO

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Attorney Docket **P1085R6**

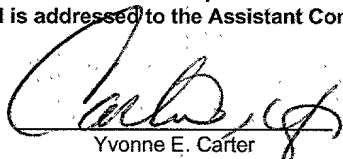
PATENT

**CERTIFICATION UNDER 37 CFR 1.10**

**EM168888508US**: Express Mail Number

January 21, 2000: Date of Deposit

I hereby certify that this Non-provisional Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

  
Yvonne E. Carter

jc584 U.S. PTO  
09/489394  
01/21/00

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**BOX PATENT APPLICATION**

Assistant Commissioner of Patents

Washington, D.C. 20231

**NON-PROVISIONAL APPLICATION TRANSMITTAL UNDER 37 CFR 1.53(b)**

Transmitted herewith for filing is a non-provisional patent application:

Inventor(s) (or Application "Identifier"):

**Vanessa Hsei, San Jose, CA  
Iphigenia Koumenis, Winston-Salem, NC  
Steven Leong, Berkeley, CA  
Zahra Shahrokh, San Francisco, CA  
Gerardo Zapata, Foster City, CA**

Title: **ANTIBODY FRAGMENT-POLYMER CONJUGATES AND USES OF SAME**

**1. Type of Application**

☐ This application is for an original, non-provisional application.

☒ This is a non-provisional application claiming priority to provisional application no. 60/116,787, filed 21 January 1999, the entire disclosure of which is hereby incorporated by reference.

☐ This is a ☐ continuation-in-part ☐ continuation ☐ divisional application claiming priority to application Serial Number\_\_, filed \_\_, the entire disclosure of which is hereby incorporated by reference.

**2. Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b) (Non-provisional)**

288 pages of specification  
14 pages of claims  
1 page(s) of abstract  
145 sheet(s) of drawings

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☐ formal    ☒ informal

### 3. Declaration or Oath

**(for new and CIP applications; also for Cont./Div. where inventor(s) are being added)**

  X   An executed declaration of the inventor(s) ☐ is enclosed    ☒ will follow.

**(for Cont./Div. where inventorship is the same or inventor(s) being deleted)**

       A copy of the executed declaration/oath filed in the prior application is enclosed (37 CFR 1.63(d)).

**(for Cont./Div. where inventor(s) being deleted)**

       A signed statement is attached deleting inventor(s) named in the prior application (see 37 CFR 1.63(d)(2) and 1.33(b)).

### 4. Assignment

**(for new and CIP applications)**

  X   An Assignment of the invention to GENENTECH, INC. ☐ is enclosed with attached Recordation Form Cover Sheet    ☒ will follow.

**(for cont./div.)**

       The prior application is assigned of record to Genentech, Inc.

### 5. Amendments

       Cancel in this application original claims \_\_\_ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

  X   A preliminary amendment is enclosed. Please enter the preliminary amendment to the claims before calculating the filing fee.

**6. Fee Calculation (37 CFR 1.16)**

The fee has been calculated as follows:

<b>CLAIMS FOR FEE CALCULATION</b>					
Number Filed		Number Extra		Rate	Basic Fee 37 CFR 1.16(a)
					\$690.00
Total Claims	37	- 20 =	17	X \$18.00	\$629.00
Independent Claims	1	- 3 =	0	X \$78.00	\$0.00
Multiple dependent claim(s), if any				+ \$260.00	\$0.00
<b>Filing Fee Calculation</b>					<b>\$1,319.00</b>

**7. Method of Payment of Fees**

The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of \$1,319.00. **A duplicate copy of this transmittal is enclosed.**

**8. Authorization to Charge Additional Fees**

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR §1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. **A duplicate copy of this sheet is enclosed.**

**9. Additional Papers Enclosed**

- ☐ Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations
- ☒ Submission of "Sequence Listing", computer readable copy, certificate re: sequence listing, and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.
- ☐ A new Power of Attorney or authorization of agent.
- ☐ Other:

**10. Maintenance of Copendency of Prior Application (for continuation and divisional applications)**

***[This item must be completed and the necessary papers filed in the prior application if the period set in the prior application has run]***

- \_\_\_\_\_ A petition, fee and/or response has been filed to extend the term in the pending prior application until \_\_\_\_\_
- \_\_\_\_\_ A copy of the petition for extension of time in the **prior** application is attached.

**11. Correspondence Address:**

- X Address all future communications to:

GENENTECH, INC.  
Attn: Richard B. Love  
1 DNA Way  
South San Francisco, CA 94080-4990  
(650) 225-5530

Respectfully submitted,  
GENENTECH, INC.

Date: January 21, 2000

By: Richard B. Love  
Richard B. Love  
Reg. No. 34,659

1 DNA Way  
So. San Francisco, CA 94080-4990  
Phone: (650) 225-5530  
Fax: (650) 952-9881



**6. Fee Calculation (37 CFR 1.16)**

The fee has been calculated as follows:

CLAIMS FOR FEE CALCULATION					
Number Filed		Number Extra		Rate	Basic Fee 37 CFR 1.16(a)
					\$690.00
Total Claims	37	- 20 =	17	X \$18.00	\$629.00
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Multiple dependent claim(s), if any				+ \$260.00	\$0.00
Filing Fee Calculation					\$1,319.00

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- ☐ Other:

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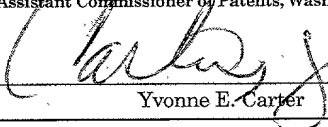
***[This item must be completed and the necessary papers filed in the prior application if the period set in the prior application has run]***

- \_\_\_\_\_ A petition, fee and/or response has been filed to extend the term in the pending prior application until
- \_\_\_\_\_ A copy of the petition for extension of time in the **prior** application is attached.

**11. Correspondence Address:**

X Address all future communications to:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<p>In re Application of</p> <p>Vanessa Hsei et al.</p> <p>Serial No.: To be assigned</p> <p>Filed: 21 January 2000</p> <p>For: ANTIBODY FRAGMENT- POLYMER CONJUGATES AND USES OF SAME</p>	<p>Group Art Unit: To be assigned</p> <p>Examiner: To be assigned</p>
	<p>CERTIFICATION UNDER 37 CFR 1.10</p> <p><b>EM168888508US: Express Mail Number</b> <b>January 21, 2000: Date of Deposit</b></p> <p>I hereby certify that this Preliminary Amendment and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.</p> <p> Yvonne E. Carter</p>

**PRELIMINARY AMENDMENT UNDER 37 C.F.R. §1.115**

Box Patent Application  
Assistant Commissioner of Patents  
Washington, D.C. 20231

Sir:

Before the calculation of fees due in connection with the filing of the above-identified application, Applicants respectfully request entry of the following amendment to the claims in the application.

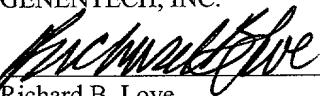
Please cancel claims 38-123.

In light of the above, Applicants believe that the application is now in condition for examination.

Respectfully submitted,

GENENTECH, INC.

By:

  
Richard B. Love  
Reg. No. 34,659

Date: January 21, 2000

One DNA Way  
So. San Francisco, CA 94080-4990  
Phone: (650) 225-5530  
Fax: (650) 952-9881

## ANTIBODY FRAGMENT-POLYMER CONJUGATES AND USES OF SAME

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This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application number 60/116,787 filed January 21, 1999, the contents of which are incorporated herein by reference.

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### FIELD OF THE INVENTION

This application relates to the field of antibody fragments derivatized with polymers, and in particular to the use of such derivatization to increase the circulation half-lives of antibody fragment-polymer conjugates. This application also relates to the use of such antibody fragment-polymer conjugates in the treatment of diseases.

### BACKGROUND

Modification of proteins with polyethylene glycol ("PEGylation") has the potential to increase residence time and reduce immunogenicity in vivo. For example, Knauf et al., J. Biol. Chem., 263: 15064-15070 (1988) reported a study of the pharmacodynamic behavior in rats of various polyoxylated glycerol and polyethylene glycol modified species of interleukin-2. Despite the known advantage of PEGylation, PEGylated proteins have not been widely exploited for clinical applications. In the case of antibody fragments, PEGylation has not been shown to extend serum half-life to useful levels. Delgado et al., Br. J. Cancer, 73: 175-182 (1996), Kitamura et al., Cancer Res., 51: 4310-4315 (1991), Kitamura et al., Biochem. Biophys. Res. Comm., 171: 1387-1394 (1990), and Pedley et al., Br. J. Cancer, 70: 1126-1130 (1994) reported studies characterizing blood clearance and tissue uptake of certain anti-tumor antigen antibodies or antibody fragments derivatized with low molecular weight (5 kD) PEG. Zapata et al., FASEB J., 9: A1479 (1995) reported that low molecular weight (5 or 10 kD) PEG attached to a

sulfhydryl group in the hinge region of a Fab' fragment reduced clearance compared to the parental Fab' molecule.

It is now well established that angiogenesis is implicated in the pathogenesis of a variety of disorders. These include solid tumors, intraocular neovascular syndromes such as proliferative retinopathies or age-related macular degeneration (AMD), rheumatoid arthritis, and psoriasis (Folkman *et al. J. Biol. Chem.* 267:10931-10934 (1992); Klagsbrun *et al. Annu. Rev. Physiol.* 53:217-239 (1991); and Garner A, *Vascular diseases. In: Pathobiology of ocular disease. A dynamic approach.* Garner A, Klintworth GK, Eds. 2nd Edition Marcel Dekker, NY, pp 1625-1710 (1994)). In the case of solid tumors, the neovascularization allows the tumor cells to acquire a growth advantage and proliferative autonomy compared to the normal cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several other tumors (Weidner *et al. N Engl J Med* 324:1-6 (1991); Horak *et al. Lancet* 340:1120-1124 (1992); and Macchiarini *et al. Lancet* 340:145-146 (1992)).

Work done over the last several years has established the key role of vascular endothelial growth factor (VEGF) in the regulation of normal and abnormal angiogenesis (Ferrara *et al. Endocr. Rev.* 18:4-25 (1997)). The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system (Ferrara *et al.*). Furthermore, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders (Ferrara *et al.*). The VEGF mRNA is overexpressed by the majority of human tumors examined (Berkman *et al. J Clin Invest* 91:153-159 (1993); Brown *et al. Human Pathol.* 26:86-91 (1995); Brown *et al. Cancer Res.* 53:4727-4735 (1993); Mattern *et al. Brit. J. Cancer.* 73:931-934 (1996); and Dvorak *et al. Am J. Pathol.* 146:1029-1039 (1995)). Also, the concentration of VEGF in eye fluids are highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies (Aiello *et al. N. Engl. J. Med.* 331:1480-1487 (1994)). Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by AMD (Lopez *et al. Invest. Ophthalmol.*

*Vis. Sci.* 37:855-868 (1996)). Anti-VEGF neutralizing antibodies suppress the growth of a variety of human tumor cell lines in nude mice (Kim *et al.* *Nature* 362:841-844 (1993); Warren *et al.* *J. Clin. Invest.* 95:1789-1797 (1995); Borgström *et al.* *Cancer Res.* 56:4032-4039 (1996); and Melnyk *et al.* *Cancer Res.* 56:921-924 (1996)) and also inhibit intraocular angiogenesis in models of ischemic retinal disorders (Adamis *et al.* *Arch. Ophthalmol.* 114:66-71 (1996)). Therefore, anti-VEGF monoclonal antibodies or other inhibitors of VEGF action are promising candidates for the treatment of solid tumors and various intraocular neovascular disorders.

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human *erbB2* gene (also known as *HER2*, or *c-erbB-2*), which encodes a 185-kd transmembrane glycoprotein receptor (p185<sup>HER2</sup>) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon *et al.*, *Science* 235:177-182 [1987]; Slamon *et al.*, *Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore *et al.*, *Science* 237:78-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy *et al.*, *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human *erbB2* protein products and proteins encoded by the rat equivalent of the *erbB2* gene (*neu*) have been described. Drebin *et al.*, *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat *neu* gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the *neu* protooncogene) and inhibits colony formation of these cells. In Drebin *et al.* *PNAS (USA)* 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of *neu*-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the *neu* oncogene was initially isolated) implanted into nude mice. Drebin *et al.* in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat

*neu* gene product. All of the antibodies were found to exert a cytostatic effect on the growth of *neu*-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant *in vitro* lysis of *neu*-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the *neu*-transformed cells. Drebin *et al.* *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on *neu*-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-*neu* antibodies are reviewed in Myers *et al.*, *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published October 13, 1994.

Hudziak *et al.*, *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak *et al.* conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize p185<sup>erbB2</sup>-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-. See also WO89/06692 published July 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak *et al.* are further characterized in Fendly *et al.* *Cancer Research* 50:1550-1558 (1990); Kotts *et al.* *In Vitro* 26(3):59A (1990); Sarup *et al.* *Growth Regulation* 1:72-82 (1991); Shepard *et al.* *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar *et al.* *Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis *et al.* *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras *et al.* *Oncogene* 9:1829-1838 (1994); Vitetta *et al.* *Cancer Research* 54:5301-5309 (1994); Sliwkowski *et al.* *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott *et al.* *J. Biol. Chem.* 266:14300-5 (1991); and D'souza *et al.* *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

Tagliabue *et al. Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth *in vitro*.

McKenzie *et al. Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier *et al. Cancer Res.* 51:5361-5369 (1991)). Bacus *et al. Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the *erbB2* gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski *et al. PNAS (USA)* 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the *erbB2* gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis *in vitro* via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus *et al. Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus *et al.* (1990) and Stancovski *et al.* of the preceding paragraphs. Extending the i.p. studies of Stancovski *et al.*, the effect of the antibodies after i.v. injection into nude mice

harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus *et al.* also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus *et al.* saw a correlation between tumor inhibition *in vivo* and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu *et al. Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan 6, 1994 and Kasprzyk *et al. Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock *et al. Cancer Res.* 51:4575-4580 (1991); Shawver *et al. Cancer Res.* 54:1367-1373 (1994); Arteaga *et al. Cancer Res.* 54:3758-3765 (1994); and Harwerth *et al. J. Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2 or HERCEPTIN<sup>®</sup>) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anticancer therapy. (Baselga *et al., J. Clin. Oncol.* 14:737-744 [1996]).

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon *et al.*, [1987] and [1989], *supra*; Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198:165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga *et al., Oncology* 11(3 Suppl 1):43-48



[1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (*Ibid*). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga *et al.*, *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

Lymphocyte adherence to endothelium is a key event in the process of inflammation. There are at least three known pathways of lymphocyte adherence to endothelium, depending on the activation state of the T cell and the endothelial cell. T cell immune recognition requires the contribution of the T cell receptor as well as adhesion receptors, which promote attachment of T cells to antigen-presenting cells and transduce regulatory signals for T cell activation. The lymphocyte function associated (LFA) antigen-1 (LFA-1, CD11a,  $\alpha$ -chain/CD18,  $\beta$ -chain) has been identified as the major integrin receptor on lymphocytes involved in these cell adherence interactions leading to several pathological states. ICAM-1, the endothelial cell immunoglobulin-like adhesion molecule, is a known ligand for LFA-1 and is implicated directly in graft rejection, psoriasis, and arthritis.

LFA-1 is required for a range of leukocyte functions, including lymphokine production of helper T cells in response to antigen-presenting cells, killer T cell-mediated target cell lysis, and immunoglobulin production through T cell-B cell interactions. Activation of antigen receptors on T cells and B cells allows LFA-1 to bind its ligand with higher affinity.

Monoclonal antibodies (MAbs) directed against LFA-1 led to the initial identification and investigation of the function of LFA-1. Davignon *et al.*, *J. Immunol.*, 127: 590 (1981). LFA-1 is present only on leukocytes [Krensky *et al.*, *J. Immunol.*, 131: 611 (1983)], and ICAM-1 is distributed on activated leukocytes, dermal fibroblasts, and endothelium. Dustin *et al.*, *J. Immunol.*, 137: 245 (1986).

Previous studies have investigated the effects of anti-CD11a MAbs on many T-cell-dependent immune functions *in vitro* and a limited number of immune responses *in vivo*. *In vitro*, anti-CD11a MAbs inhibit T-cell activation [Kuypers *et al.*, *Res. Immunol.*, 140: 461

(1989)], T-cell-dependent B-cell proliferation and differentiation [Davignon *et al.*, *supra*; Fischer *et al.*, *J. Immunol.*, 136: 3198 (1986)], target cell lysis by cytotoxic T lymphocytes [Krensky *et al.*, *supra*], formation of immune conjugates (Sanders *et al.*, *J. Immunol.*, 137: 2395 (1986); Mentzer *et al.*, *J. Immunol.*, 135: 9 (1985)), and the adhesion of T-cells to vascular endothelium.

5 Lo *et al.*, *J. Immunol.*, 143: 3325 (1989). Also, the antibody 5C6 directed against CD11b/CD18 was found to prevent intra-islet infiltration by both macrophages and T cells and to inhibit development of insulin-dependent diabetes mellitus in mice. Hutchings *et al.*, *Nature*, 348: 639 (1990).

IgE is a member of the immunoglobulin family that mediates allergic responses such as asthma, food allergies, and other type 1 hypersensitivity reactions. IgE is secreted by and expressed on the surface of B-cells or B-lymphocytes. IgE binds to B-cells (as well as monocytes, eosinophils and platelets) through its Fc region to a low affinity IgE receptor (Fc<sub>ε</sub>RII). Upon exposure of a mammal to an allergen, B-cells bearing a membrane-bound IgE antibody specific for the antigen are activated to form IgE-secreting plasma cells. The allergen-specific, soluble IgE secreted by plasma cells circulates through the bloodstream and binds to the surface of mast cells in tissues and basophils in the blood, through the high affinity IgE receptor (Fc<sub>ε</sub>RI). The mast cells and basophils thereby become sensitized for the allergen. Subsequent exposure to the allergen results in cross linking of allergen-specific IgE bound to basophilic and mast cellular Fc<sub>ε</sub>RI, which induces a release of histamine, leukotrienes and platelet activating factors, eosinophil and neutrophil chemotactic factors and the cytokines IL-3, IL-4, IL-5 and GM-CSF, which are responsible for clinical hypersensitivity and anaphylaxis.

The pathological condition *hypersensitivity* is characterized by an excessive immune response to (an) allergen(s) resulting in gross tissue changes if the allergen is present in relatively large amounts or if the humoral and cellular immune state is at a heightened level.

Physiological changes in *anaphylactic hypersensitivity* can include intense constriction of the bronchioles and bronchi of the lungs, contraction of smooth muscle and dilation of capillaries. Predisposition to this condition appears to result from an interaction between genetic and environmental factors. Common environmental allergens which induce anaphylactic

hypersensitivity are found in pollen, foods, house dust mites, animal danders, fungal spores and insect venoms. Atopic allergy is associated with anaphylactic hypersensitivity and includes disorders such as asthma, allergic rhinitis and conjunctivitis (hay fever), eczema, urticaria and food allergies. Anaphylactic shock, a dangerous life-threatening condition that can occur in the progression of anaphylaxis, is usually provoked by insect stings or parenteral medication.

Recently, a treatment strategy has been pursued for Type 1 hypersensitivity or anaphylactic hypersensitivity which blocks IgE from binding to the high-affinity receptor (Fc $\epsilon$ RI) found on basophils and mast cells, and thereby prevents the release of histamine and other anaphylactic factors resulting in the pathological condition.

Interleukin-8 (IL-8) is neutrophil chemotactic peptide secreted by a variety of cells in response to inflammatory mediators (for a review see Hebert *et al.* Cancer Investigation 11(6):743 (1993)). IL-8 can play an important role in the pathogenesis of inflammatory disorders, such as adult respiratory distress syndrome (ARDS), septic shock, and multiple organ failure. Immune therapy for such inflammatory disorders can include treatment of an affected patient with anti-IL-8 antibodies.

Sticherling *et al.* (J. Immunol. 143:1628 (1989)) disclose the production and characterization of four monoclonal antibodies against IL-8. WO 92/04372, published March 19, 1992, discloses polyclonal antibodies which react with the receptor-interacting site of IL-8 and peptide analogs of IL-8, along with the use of such antibodies to prevent an inflammatory response in patients. St. John *et al.* (Chest 103:932 (1993)) review immune therapy for ARDS, septic shock, and multiple organ failure, including the potential therapeutic use of anti-IL-8 antibodies. Sekido *et al.* (Nature 365:654 (1993)) disclose the prevention of lung reperfusion injury in rabbits by a monoclonal antibody against IL-8. Mulligan *et al.* (J. Immunol. 150:5585 (1993)), disclose protective effects of a murine monoclonal antibody to human IL-8 in inflammatory lung injury in rats.

WO 95/23865 (International Application No. PCT/US95/02589 published September 8, 1995) demonstrates that anti-IL-8 monoclonal antibodies can be used therapeutically in the treatment of other inflammatory disorders, such as bacterial pneumonias and inflammatory bowel

disease.

Anti-IL-8 antibodies are additionally useful as reagents for assaying IL-8. For example, Sticherling *et al.* (*Arch. Dermatol. Res.* 284:82 (1992)), disclose the use of anti-IL-8 monoclonal antibodies as reagents in immunohistochemical studies. Ko *et al.* (*J. Immunol. Methods* 149:227 (1992)) disclose the use of anti-IL-8 monoclonal antibodies as reagents in an enzyme-linked immunoabsorbent assay (ELISA) for IL-8.

### SUMMARY OF THE INVENTION

One aspect of the invention is a conjugate consisting essentially of one or more antibody fragments covalently attached to one or more nonproteinaceous polymer molecules, wherein the apparent size of the conjugate is at least about 500 kD.

Another aspect of the invention is a conjugate formed by one or more antibody fragments covalently attached to one or more nonproteinaceous polymer molecules, wherein the apparent size of the conjugate is at least about 500 kD, and wherein the covalent structure of the conjugate is free of any matter other than the antibody fragment and nonproteinaceous polymer molecules.

Yet another aspect of the invention is a conjugate formed by the one or more antibody fragments covalently attached to one or more nonproteinaceous polymer molecules, wherein the covalent structure of the conjugate further incorporates one or more nonproteinaceous labels, wherein the covalent structure of the conjugate is free of any matter other than the antibody fragment, nonproteinaceous polymer and nonproteinaceous label molecules, and wherein the apparent size of the conjugate is at least about 500 kD.

Still another aspect of the invention is a conjugate consisting essentially of one or more antibody fragments covalently attached to one or more nonproteinaceous polymer molecules, wherein the apparent size of the conjugate is at least about 500 kD, and wherein at least one antibody fragment comprises an antigen binding site that binds to a polypeptide selected from the group consisting of human vascular endothelial growth factor (VEGF), human p185 receptor-like tyrosine kinase (HER2), human CD20, human CD18, human CD11a, human IgE, human apoptosis receptor-2 (Apo-2), human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), human tissue factor,

human  $\alpha_4\beta_7$  integrin, human GPIIb-IIIa integrin, human epidermal growth factor receptor (EGFR), human CD3, and human interleukin-2 receptor  $\alpha$ -chain (TAC).

### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graph depicting the blocking of IL-8 mediated elastase release from neutrophils by anti-IL-8 monoclonal antibody 5.12.14.

Figure 2 is a graph depicting the inhibition of  $^{125}\text{I}$ -IL-8 binding to neutrophils by unlabeled IL-8.

Figure 3 demonstrates that a isotype matched negative control Fab (denoted as "4D5 Fab") does not inhibit the binding of  $^{125}\text{I}$ -IL-8 to human neutrophils.

Figure 4 is a graph depicting the inhibition of binding of  $^{125}\text{I}$ -IL-8 to human neutrophils by chimeric 5.12.14 Fab with an average  $\text{IC}_{50}$  of 1.6 nM.

Figure 5 is a graph depicting the inhibition of binding of  $^{125}\text{I}$ -IL-8 to human neutrophils by chimeric 6G.4.25 Fab with an average  $\text{IC}_{50}$  of 7.5 nM.

Figure 6 demonstrates the inhibition of human IL-8 mediated neutrophil chemotaxis by chimeric 6G4.2.5 Fab and chimeric 5.12.14 Fab.

Figure 7 demonstrates the relative abilities of chimeric 6G4.2.5 Fab and chimeric 5.12.14 Fab to inhibit rabbit IL-8 mediated neutrophil chemotaxis.

Figure 8 depicts the stimulation of elastase release from human neutrophils by various concentrations of human and rabbit IL-8. The relative extent of elastase release was quantitated by measurement of absorbance at 405 nm. The data represent mean  $\pm$  SEM of triplicate samples.

Figure 9 is a graph depicting the ability of chimeric 6G4.2.5 Fab and chimeric 5.12.14 Fab to inhibit elastase release from human neutrophils stimulated by human IL-8. The results were normalized to reflect the percentage of elastase release elicited by 100 nM IL-8 alone. The data represent the mean  $\pm$  SEM of three separate experiments performed on different days with different blood donors.  $\text{IC}_{50}$  values were calculated by four parameter fit.

Figure 10 is a graph depicting the relative abilities of chimeric 6G4.2.5 Fab and chimeric 5.12.14 Fab to inhibit elastase release from human neutrophils stimulated by rabbit IL-8. The

results were normalized to reflect the percentage of elastase release elicited by 100 nM IL-8 alone. The data represent the mean  $\pm$  SEM of three separate experiments performed on different days with different blood donors. IC<sub>50</sub> values were calculated by four parameter fit.

Figures 11A-11J are a set of graphs depicting the following parameters in a rabbit ulcerative colitis model: Figure 11A depicts myeloperoxidase levels in tissue; Figure 11B depicts IL-8 levels in tissue; Figure 11C depicts colon weight; Figure 11D depicts gross inflammation; Figure 11E depicts edema; Figure 11F depicts extent of necrosis; Figure 11G depicts severity of necrosis; Figure 11H depicts neutrophil margination; Figure 11I depicts neutrophil infiltration; and Figure 11J depicts mononuclear infiltration.

Figure 12 is a graph depicting the effect of anti-IL-8 monoclonal antibody treatment on the number of neutrophils in bronchoalveolar lavage (BAL) fluid in animals infected with Streptococcus pneumoniae, Escherichia coli, or Pseudomonas aeruginosa. Treatment with 6G4.2.5 significantly reduced the number of neutrophils present in the BAL fluid compared to animals treated with isotype control mouse IgG (Figure 12).

Figure 13 depicts the DNA sequences (SEQ ID NOS: 1-6) of three primers designed for each of the light and heavy chains. Multiple primers were designed in order to increase the chances of primer hybridization and efficiency of first strand cDNA synthesis for cloning the variable light and heavy regions of monoclonal antibody 5.12.14.

Figure 14 depicts the DNA sequences (SEQ ID NOS: 7-10) of one forward primer and one reverse primer for the 5.12.14 light chain variable region amplification.

Figure 15 depicts the DNA sequences (SEQ ID NOS: 11-15) of one forward primer and one reverse primer for the 5.12.14 heavy chain variable region amplification.

Figure 16 depicts the DNA sequence (SEQ ID NO: 16) and the amino acid sequence (SEQ ID NO: 17) of the 5.12.14 light chain variable region and partial murine constant light region. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). Important restriction sites are indicated in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable light region is amino acids 1 to 109. The partial murine constant light region is amino acids 110

to 123 (in italics).

Figure 17 depicts the DNA sequence (SEQ ID NO: 18) and the amino acid sequence (SEQ ID NO: 19) of the 5.12.14 heavy chain variable region and partial murine constant heavy region. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). Important restriction sites are indicated in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable heavy region is amino acids 1 to 120. The partial murine constant heavy region is amino acids 121 to 130.

Figure 18 depicts the DNA sequences (SEQ ID NOS: 20-23) of amplification primers used to convert murine light and heavy chain constant region residues to their human equivalents.

Figure 19 depicts the DNA sequence (SEQ ID NO: 24) and the amino acid sequence (SEQ ID NO: 25) for the 5.12.14 light chain variable region and the human IgG1 light chain constant region. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). The human constant region is denoted in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable light region is amino acids 1 to 109. The human constant light region is amino acids 110 to 215.

Figures 20A-20B depict the DNA sequence (SEQ ID NO: 26) and the amino acid sequence (SEQ ID NO: 27) for the 5.12.14 heavy chain variable region and the heavy chain constant region of human IgG1. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). The human constant region is denoted in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable heavy region is amino acids 1 to 120. The human constant heavy region is amino acids 121 to 229.

Figure 21 depicts the DNA sequences (SEQ ID NOS: 1-6) of three primers designed for each of the light and heavy chains. Multiple primers were designed in order to increase the chances of primer hybridization and efficiency of first strand cDNA synthesis for cloning the variable light and heavy regions of monoclonal antibody 6G4.2.5.

Figure 22 depicts the DNA sequences (SEQ ID NOS: 28-31) of one forward primer and one reverse primer for the 6G4.2.5 light chain variable region amplification.

Figure 23 depicts the DNA sequences (SEQ ID NOS: 32,33,11,15,14, and 13) of one forward primer and one reverse primer for the 6G4.2.5 heavy chain variable region amplification.

Figure 24 depicts the DNA sequence (SEQ ID NO: 34) and the amino acid sequence (SEQ ID NO: 35) of the 6G4.2.5 light chain variable region and partial murine constant light region. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). Useful cloning sites are in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable light region is amino acids 1 to 114. The partial murine constant light region is amino acids 115 to 131.

Figure 25 depicts the DNA sequence (SEQ ID NO: 36) and the amino acid sequence (SEQ ID NO: 37) of the 6G4.2.5 heavy chain variable region and partial murine constant heavy region. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). Useful cloning sites are in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable heavy region is amino acids 1 to 122. The partial murine constant heavy region is amino acids 123 to 135.

Figure 26 depicts the DNA sequences (SEQ ID NOS: 38-40) of primers to convert the murine light chain and heavy chain constant regions to their human equivalents.

Figures 27A-27B depict the DNA sequence (SEQ ID NO: 41) and the amino acid sequence (SEQ ID NO: 42) for the chimeric 6G4.2.5 light chain. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). The human constant region is denoted in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable light region is amino acids 1 to 114. The human constant light region is amino acids 115 to 220.

Figures 28A-28B depict the DNA sequence (SEQ ID NO: 43) and the amino acid sequence (SEQ ID NO: 44) for the chimeric 6G4.2.5 heavy chain. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). The human constant region is denoted in italics. The signal peptide of



STII is amino acids -23 to -1. The murine variable heavy region is amino acids 1 to 122. The human constant heavy region is amino acids 123 to 231.

Fig. 29 depicts an amino acid sequence alignment of murine 6G425 light chain variable domain (SEQ ID NO: 45), humanized 6G425 F(ab)-1 light chain variable domain (SEQ ID NO: 46), and human light chain  $\kappa$ I consensus framework (SEQ ID NO: 47) amino acid sequences, and an amino acid sequence alignment of murine 6G425 heavy chain variable domain (SEQ ID NO: 48), humanized 6G425 F(ab)-1 heavy chain variable domain (SEQ ID NO: 49), and human IgG1 subgroup III heavy chain variable domain (SEQ ID NO: 50) amino acid sequences, used in the humanization of 6G425. Light chain CDRs are labeled L1, L2, L3; heavy chain CDRs are labeled H1, H2, and H3. = and + indicate CDR sequences as defined by X-ray crystallographic contacts and sequence hypervariability, respectively. # indicates a difference between the aligned sequences. Residue numbering is according to Kabat *et al.* Lower case lettering denotes the insertion of an amino acid residue relative to the humIII consensus sequence numbering.

Figs. 30A, 30B and 30C are graphs depicting the ability of F(ab)-9 (humanized 6G4V11 Fab) to inhibit human wild type IL-8, human monomeric IL-8, and rhesus IL-8 mediated neutrophil chemotaxis, respectively. Fig. 30A presents inhibition data for F(ab)-9 samples at concentrations of 0.06 nM, 6.25 nM, 12.5 nM, 25 nM, 50 nM, and 100 nM, for an isotype control antibody (denoted "4D5") sample at a concentration of 100 nM, and for a no antibody control sample, in the presence of 2nM human wild type IL-8. Fig. 30B presents inhibition data for F(ab)-9 samples at concentrations of 6.25 nM, 12.5 nM, 25 nM, and 50 nM, for an isotype control antibody (denoted "4D5") sample at a concentration of 100 nM, and for a no antibody control sample, in the presence of 4 nM human monomeric IL-8 (denoted as "BD59" and as "monomeric IL-8"). Fig. 30C presents inhibition data for F(ab)-9 samples at concentrations of 1 nM, 12.5 nM, 25 nM, and 50 nM, for an isotype control antibody (denoted "4D5") sample at a concentration of 100 nM, and for a no antibody control sample, in the presence of 2 nM rhesus IL-8. In addition, Figs. 30A-30C each presents data for a no IL-8 buffer control sample (denoted as "Buffer") in the respective inhibition assay.

Fig. 31A depicts the amino acid sequences of the humanized anti-IL-8 6G4.2.5V11 light

chain in an N-terminal fusion with the STII leader peptide (SEQ ID NO: 51), the humanized anti-IL-8 6G4.2.5V11 heavy chain in an N-terminal fusion with the STII leader peptide (SEQ ID NO: 52), and a peptide linker in a C-terminal fusion with M13 phage gene-III coat protein (SEQ ID NO: 53).

Fig. 31B depicts the nucleic acid sequence (SEQ ID NO: 54) and the translated amino acid sequence (SEQ ID NO: 51) of the humanized anti-IL-8 6G4.2.5V11 light chain in an N-terminal fusion with the STII leader peptide.

Fig. 31C depicts the amino acid sequences of the humanized anti-IL-8 6G4.2.5V19 light chain in an N-terminal fusion with the STII leader peptide (SEQ ID NO: 51), and the humanized anti-IL-8 6G4.2.5V19 heavy chain in an N-terminal fusion with the STII leader peptide (SEQ ID NO: 55).

Fig. 32 is a three dimensional computer model of the humanized anti-IL-8 6G4.2.5V11 antibody. Heavy chain CDR loops and variable domain regions appear in purple, and CDR-H3 side chain residues appear in yellow. Heavy chain constant domain regions appear in red. Light chain CDR loops and variable domain regions appear in off-white, and the Asn residue at amino acid position 35 (N35) in CDR L1 appears in green. Light chain constant domain regions appear in amber.

Fig. 33 is a Scatchard plot depicting the inhibition of <sup>125</sup>I-IL-8 binding to human neutrophils exhibited by intact murine 6G4.2.5 antibody (denoted 6G4 murine mAb), 6G4.2.5 murine-human chimera Fab (denoted 6G4 chimera), humanized 6G4.2.5 Fab versions 1 and 11 (denoted V1 and V11), and variant 6G4.2.5V11N35A Fab (denoted V11N35A).

Figs. 34A, 34B, 34C and 34D are graphs depicting the ability of 6G4.2.5V11N35A Fab to inhibit human wild type IL-8, human monomeric IL-8, rabbit IL-8, and rhesus IL-8 mediated neutrophil chemotaxis, respectively. Fig. 34A presents inhibition data for 6G4.2.5V11N35A Fab samples at concentrations of 0.5, 1, 2, 4, 8, 16, and 33 nM, for an isotype control antibody (denoted "4D5") sample at a concentration of 33 nM, and for a no antibody control (denoted "HuIL-8") sample, in the presence of 2 nM human wild type IL-8. Fig. 34B presents inhibition data for 6G4.2.5V11N35A Fab samples at concentrations of 0.5, 1, 2, 4, 8, 16, and 33 nM, for an

intact 6G4.2.5 mAb sample at a concentration of 33 nM, for an isotype control antibody (denoted as "4D5") sample at a concentration of 33 nM, and for a no antibody control (denoted "BD59") sample, in the presence of 2 nM human monomeric IL-8. Fig. 34C presents inhibition data for 6G4.2.5V11N35A Fab samples at concentrations of 0.5, 1, 2, 4, 8, 16, and 33 nM, for an intact 6G4.2.5 mAb sample at a concentration of 33 nM, for an isotype control antibody (denoted "4D5") sample at a concentration of 33 nM, and for a no antibody control (denoted "Rab IL-8") sample, in the presence of 2 nM rabbit IL-8. Fig. 34D presents inhibition data for 6G4.2.5V11N35A Fab samples at concentrations of 0.5, 1, 2, 4, 8, 16, and 33 nM, for an intact 6G4.2.5 mAb sample at a concentration of 33 nM, for an isotype control antibody (denoted as "4D5") sample at a concentration of 33 nM, and for a no antibody control (denoted "Rhe IL-8") sample, in the presence of 2 nM rhesus IL-8. In addition, Figs. 34B-34D each presents data for human wild type IL-8 control (denoted "HuIL-8") samples at a concentration of 2 nM in the respective assay, and Figs. 34A-34D each presents data for a no IL-8 buffer control (denoted "Buffer") sample in the respective assay.

Fig. 35 depicts the amino acid sequences of the humanized anti-IL-8 6G4.2.5V11N35A light chain in an N-terminal fusion with the STII leader peptide (SEQ ID NO: 56), the humanized anti-IL-8 6G4.2.5V11N35A heavy chain in an N-terminal fusion with the STII leader peptide (SEQ ID NO: 52), and the GCN4 leucine zipper peptide (SEQ ID NO: 57). The Ala residue (substituted for the wild type Asn residue) at amino acid position 35 in the 6G4.2.5V11N35A light chain appears in bold case. A putative pepsin cleavage site in the GCN4 leucine zipper sequence is underlined.

Fig. 36 depicts the DNA sequence (SEQ ID NO: 58) and the amino acid sequence (SEQ ID NO: 56) of the humanized anti-IL-8 6G4.2.5V11N35A light chain in an N-terminal fusion with the STII leader peptide. Complementarity determining regions L1, L2, and L3 are underlined

Figs. 37A-37B depict the DNA sequence (SEQ ID NO: 59) and the amino acid sequence (SEQ ID NO: 60) of the humanized anti-IL-8 6G4.2.5V11N35A heavy chain in an N-terminal fusion with the STII leader peptide and in a C-terminal fusion with the GCN4 leucine zipper

sequence. Complementarity determining regions H1, H2, and H3 are underlined.

Fig. 38 is a Scatchard plot depicting the inhibition of  $^{125}\text{I}$ -IL-8 binding to human neutrophils exhibited by 6G4.2.5V11N35A Fab (denoted Fab), 6G4.2.5V11N35A F(ab')<sub>2</sub> (denoted F(ab')<sub>2</sub>), and human wild type IL-8 control (denoted IL-8).

Fig. 39 is a graph depicting a comparison of the wild type human IL-8 mediated neutrophil chemotaxis inhibition activities of the 6G4.2.5V11N35A F(ab')<sub>2</sub> and 6G4.2.5V11N35A Fab. Inhibition data are presented for 6G4.2.5V11N35A Fab samples (denoted "N35A Fab") and 6G4.2.5V11N35A F(ab')<sub>2</sub> samples (denoted N35A F(ab')<sub>2</sub>) at concentrations of 0.3, 1, 3, 10, 30, and 100 nM, for an isotype control antibody (denoted as "4D5") sample at a concentration of 100 nM, and for a no antibody control sample, in the presence of 2 nM human wild type IL-8. In addition, inhibition data are presented for no IL-8 buffer control samples (denoted "Buffer").

Fig. 40 is a graph depicting the ability of 6G4.2.5V11N35A F(ab')<sub>2</sub> to inhibit human monomeric IL-8, rhesus IL-8, and rabbit IL-8 mediated neutrophil chemotaxis. Human monomeric IL-8 mediated neutrophil chemotaxis data are presented for 6G4.2.5V11N35A F(ab')<sub>2</sub> samples at concentrations of 0.3, 1, 3, and 10 nM, for an isotype control antibody (denoted as "4D5") sample at a concentration of 100 nM, and for a no antibody control sample (denoted as "BD59"), in the presence of human monomeric IL-8 (denoted as "BD59") at a concentration of 0.5 nM. Rhesus IL-8 mediated neutrophil chemotaxis data are presented for 6G4.2.5V11N35A F(ab')<sub>2</sub> samples at concentrations of 0.3, 1, 3, and 10 nM, and for a no antibody control sample, in the presence of rhesus IL-8 at a concentration of 2 nM. Rabbit IL-8 mediated neutrophil chemotaxis data are presented for 6G4.2.5V11N35A F(ab')<sub>2</sub> samples at concentrations of 0.3, 1, 3, and 10 nM, and for a no antibody control sample, in the presence of rabbit IL-8 at a concentration of 2 nM. In addition, inhibition data are presented for a no IL-8 buffer control sample (denoted as "Buffer") and for a 2 nM human wild type IL-8 (denoted as "HuIL-8").

Figs. 41A-41V depict the nucleic acid sequence (SEQ ID NO: 61) of the p6G4V11N35A.F(ab')<sub>2</sub> vector.

Fig. 42 depicts the nucleic acid sequences of the stop template primer (SEQ ID NO: 63) and the NNS randomization primer (SEQ ID NO: 64) used for random mutagenesis of amino acid position 35 in variable light chain CDR-L1 of humanized antibody 6G4V11.

Fig. 43A is a table of data describing the frequencies of different phage display clones obtained from the randomization of amino acid position 35 in variable light chain CDR-L1 of humanized antibody 6G4V11.

Figs. 43B, 43C, 43D and 43E are graphs of displacement curves depicting the inhibition of <sup>125</sup>I-IL-8 binding to neutrophils exhibited by the 6G4V11N35A, 6G4V11N35D, 6G4V11N35E and 6G4V11N35G Fab's.

Fig. 44 contains a graph depicting the typical kinetics of an anti-IL-8 antibody fragment (6G4V11N35A F(ab')<sub>2</sub>) binding to IL-8. Fig. 44 also contains a table of data providing the equilibrium constant for 6G4V11N35A Fab binding to IL-8 (rate constants were not determined "ND"), and the equilibrium and rate constants for 6G4V11N35A F(ab')<sub>2</sub> and 6G4V11N35E Fab binding to IL-8.

Fig. 45 depicts the DNA sequence (SEQ ID NO: 65) and amino acid sequence (SEQ ID NO: 62) of the 6G4V11N35E light chain in an N-terminal fusion with the STII leader peptide. Complementarity determining regions L1, L2 and L3 are underlined.

Fig. 46 is a graph depicting the ability of 6G4V11N35E Fab to inhibit human IL-8 (dark columns) and rabbit IL-8 (light columns) mediated neutrophil chemotaxis. Data are presented for 6G4V11N35E Fab samples at concentrations of 0.4, 1.2, 3.7, 11 and 33 nM, and for an isotype control antibody (4D5) sample at a concentration of 100 nM, in the presence of 2 nM human IL-8 or 2 nM rabbit IL-8. In addition, inhibition data are presented for a no IL-8 buffer control sample (denoted "Buffer") and for human and rabbit IL-8 control samples (denoted "IL-8").

Fig. 47 depicts the DNA sequence of the sense (SEQ ID NO: 66) and anti-sense (SEQ ID NO: 67) strands of a PvuII-XhoI synthetic nucleotide encoding amino acids Leu4 to Phe29 of the 6G4V11N35A heavy chain.

Figs. 48A-48T depict the DNA sequence (SEQ ID NO: 68) of plasmid

p6G4V11N35A.choSD9.

Figs. 49A, 49B, 49C and 49D are graphs of displacement curves depicting the inhibition of  $^{125}\text{I}$ -IL-8 binding to neutrophils exhibited by IL-8 control, intact murine 6G4.2.5 antibody, the full length IgG1 form of variant 6G4V11N35A, and the full length IgG1 form of variant 6G4V11N35E, respectively.

Figs. 50A-50B are graphs depicting the ability of full length 6G4V11N35A IgG1 and 6G4V11N35E IgG1 to inhibit human IL-8 (Fig. 50A) and rabbit IL-8 (Fig. 50B) mediated neutrophil chemotaxis.

Fig. 51 contains a graph depicting the typical kinetics of a full length anti-IL8 antibody (6G4V11N35A IgG1) binding to IL-8. Fig. 51 also contains a table of data providing the equilibrium and rate constants for full length murine 6G4.2.5 IgG2a, 6G4V11N35A IgG1 and 6G4V11N35E IgG1 binding to IL-8.

Figs. 52A and 52B are graphs of displacement curves depicting the results of an unlabeled IL-8/ $^{125}\text{I}$ -IL-8 competition radioimmunoassay performed with full length 6G4V11N35A IgG1 and 6G4V11N35E IgG1, respectively.

Fig. 53 depicts the DNA sequence (SEQ ID NO: 69) and amino acid sequence (SEQ ID NO: 70) of the 6G4V11N35A Fab' heavy chain (6G4V11N35A Fab heavy chain modified to contain a cysteine residue in the hinge region).

Figs. 54A-54C contain graphs of displacement curves depicting the IL-8 binding and  $\text{IC}_{50}$ 's for PEG-maleimide modified 6G4V11N35A Fab' molecules.

Figs. 55A-55C are graphs depicting the ability of PEG-maleimide modified 6G4V11N35A Fab' molecules to inhibit human IL-8 and rabbit IL-8 mediated neutrophil chemotaxis.

Figs. 56A-56C are graphs depicting the ability of PEG-maleimide modified 6G4V11N35A Fab' molecules to inhibit IL-8 mediated release of  $\beta$ -glucuronidase from neutrophils.

Figs. 57A-57B contain graphs of displacement curves depicting the inhibition of  $^{125}\text{I}$ -IL-8 binding to neutrophils exhibited by PEG-succinimide modified 6G4V11N35A Fab'<sub>2</sub> molecules.

Figs. 58A-58B are graphs depicting the ability of PEG-succinimide modified 6G4V11N35A F(ab')<sub>2</sub> molecules to inhibit human IL-8 mediated neutrophil chemotaxis.

Figs. 59A-59B are graphs depicting the ability of PEG-succinimide modified 6G4V11N35A F(ab')<sub>2</sub> molecules to inhibit human IL-8 mediated release of β-glucuronidase from neutrophils.

Fig. 60 is a graph depicting the theoretical molecular weight (dotted bars) and effective size (solid bars) of PEG-maleimide modified 6G4V11N35A Fab' molecules as determined by SEC-HPLC.

Figs. 61A and 61B are SDS-PAGE gels depicting the electrophoretic mobility of various PEG-maleimide modified 6G4V11N35A Fab' molecules under reducing and non-reducing conditions, respectively.

Fig. 62 contains size exclusion chromatograms (SEC-HPLC) depicting the retention times and effective (hydrodynamic) sizes of various PEG-succinimide modified 6G4V11N35A F(ab')<sub>2</sub> molecules.

Fig. 63 is a graph depicting the theoretical molecular weight (open columns), effective size determined by SEC-HPLC (solid columns), and the actual molecular weight determined by SEC-light scattering (shaded columns) for various PEG-succinimide modified 6G4V11N35A F(ab')<sub>2</sub> molecules.

Fig. 64 is an SDS-PAGE gel depicting the electrophoretic mobility of various PEG-succinimide modified 6G4V11N35A F(ab')<sub>2</sub> molecules. From left to right, lane 1 contains unmodified F(ab')<sub>2</sub>, lane 2 contains F(ab')<sub>2</sub> coupled to two 40 kD branched PEG-succinimide molecules (denoted "Br(2)-40kD(N)-F(ab')<sub>2</sub>"), lane 3 contains F(ab')<sub>2</sub> coupled to one 40 kD branched PEG-succinimide molecule (denoted "Br(1)-40kD-(N)-Fab'2"), lane 4 contains a mixture of F(ab')<sub>2</sub> coupled to four 20 kD linear PEG-succinimide molecules and F(ab')<sub>2</sub> coupled to five 20 kD linear PEG-succinimide molecules (denoted "L(4+5)-20kD-(N)-Fab'2"), lane 5 contains F(ab')<sub>2</sub> coupled to one 20 kD linear PEG-succinimide molecule (denoted "L(1)-20kD-(N)-Fab'2"), and lane 6 contains molecular weight standards.

Figs. 65A and 65B are graphs comparing the serum concentration vs. time profiles of

various PEG-maleimide modified 6G4V11N35A Fab' molecules (Fig. 65A) and various PEG-succinimide modified 6G4V11N35A F(ab')<sub>2</sub> molecules (Fig. 65B) in rabbits. In Fig. 65A, "bran.(1)40K(s)Fab'" denotes 6G4V11N35A Fab' coupled to one 40 kD branched PEG-maleimide molecule, "lin.(1)40K(s)Fab'" denotes 6G4V11N35A Fab' coupled to one 40 kD linear PEG-maleimide molecule, "lin.(1)30K(s)Fab'" denotes 6G4V11N35A Fab' coupled to one 30 kD linear PEG-maleimide molecule, "lin.(1)20K(s)Fab'" denotes 6G4V11N35A Fab' coupled to one 20 kD linear PEG-maleimide molecule. In Fig. 65B, "bran.(2)40K(N)Fab'2'" denotes 6G4V11N35A F(ab')<sub>2</sub> coupled to two 40 kD branched PEG-succinimide molecules, "bran.(1)40K(N)Fab'2'" denotes 6G4V11N35A F(ab')<sub>2</sub> coupled to one 40 kD branched PEG-succinimide molecule, and "Fab'2'" denotes unmodified 6G4V11N35A F(ab')<sub>2</sub>. In both Figs. 65A and 65B, "IgG" denotes a full length IgG1 equivalent of the human-murine chimeric anti-rabbit IL-8 Fab described in Example F below.

Fig. 66 contains graphs comparing the serum concentration vs. time profiles of 6G4V11N35A Fab' coupled to one 40 kD branched PEG-maleimide molecule (denoted as "bran.(1)40K(s)Fab'"), 6G4V11N35A F(ab')<sub>2</sub> coupled to one 40 kD branched PEG-succinimide molecule (denoted as "bran.(1)40K(N)Fab'2'"), unmodified 6G4V11N35A F(ab')<sub>2</sub> (denoted as "Fab'2'"), unmodified 6G4V11N35A Fab' (denoted as "Fab'"), and a full length IgG1 (denoted as "IgG") equivalent of the human-murine chimeric anti-rabbit IL-8 Fab described in Example F below.

Fig. 67 is a graph depicting the effect of 6G4V11N35A Fab' coupled to one 40 kD branched PEG-maleimide molecule (denoted as "PEG 40 Kd") and murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 (full length IgG2a) (denoted as "6G4.2.5") on gross weight of entire lung in an ARDS rabbit model.

Fig. 68 is a graph depicting the effect of 6G4V11N35A Fab' coupled to one branched 40 kD PEG-maleimide molecule (denoted as "PEG 40 Kd") and murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 (full length IgG2a) (denoted as "6G4.2.5") on BAL total leukocyte (light columns) and polymorphonuclear cell (dark columns) counts in an ARDS rabbit model. Untreated (no therapeutics) control animal data is denoted as "Control".



Fig. 69 is a graph depicting the effect of 6G4V11N35A Fab' coupled to one branched 40 kD PEG-maleimide molecule (denoted as "PEG 40 Kd") and murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 (full length IgG2a) (denoted as "6G4.2.5") on PaO<sub>2</sub>/FiO<sub>2</sub> ratio at 24 hours- post treatment (light columns) and 48 hours post-treatment (dark columns) in an ARDS rabbit model. Untreated (no therapeutics) control animal data is denoted as "Control".

Fig. 70A is a graph depicting PaO<sub>2</sub>/FiO<sub>2</sub> ratios obtained in 100% oxygen at 24 hours after acid instillation for: (1) rabbits (n=5) treated with 7 mg/kg IV 20 kD linear PEG-6G4V11N35E Fab' at 10 minutes before and 6 hours after acid instillation, (2) rabbits (n=7) treated with 5 mg/kg IV full length IgG murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 at 10 minutes before acid instillation, (3) rabbits (n=3) treated with 5 mg/kg IV 40 kD branched PEG-6G4V11N35A Fab' at 10 minutes before acid instillation, (4) rabbits (n=2) treated with 20 mg/kg IV 40 kD branched PEG-6G4V11N35A Fab' at 10 minutes before acid instillation, and (5) rabbits (n=25) treated with 5 ml IV saline at 10 minutes before and 6 hours after acid instillation.

Fig. 70B is a graph depicting PaO<sub>2</sub>/FiO<sub>2</sub> ratios obtained in 100% oxygen at 48 hours after acid instillation for: (1) rabbits (n=5) treated with 7 mg/kg IV 20 kD linear PEG-6G4V11N35E Fab' at 10 minutes before and 6 hours after acid instillation, (2) rabbits (n=7) treated with 5 mg/kg IV full length IgG murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 at 10 minutes before acid instillation, (3) rabbits (n=3) treated with 5 mg/kg IV 40 kD branched PEG-6G4V11N35A Fab' at 10 minutes before acid instillation, (4) rabbits (n=2) treated with 20 mg/kg IV 40 kD branched PEG-6G4V11N35A Fab' at 10 minutes before acid instillation, and (5) rabbits (n=16) treated with 5 ml IV saline at 10 minutes before and 6 hours after acid instillation.

Fig. 70C is a graph depicting gross lung weight (in grams)/body weight (in kilograms) ratios (denoted as "GLW/BW Ratio") obtained at 72 hours post reperfusion for: (1) rabbits (n=5) treated with 7 mg/kg IV 20 kD linear PEG-6G4V11N35E Fab' at 10 minutes before and 6 hours after acid instillation, (2) rabbits (n=7) treated with 5 mg/kg IV full length IgG murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 at 10 minutes before acid instillation, (3) rabbits (n=3) treated

with 5 mg/kg IV 40 kD branched PEG-6G4V11N35A Fab' at 10 minutes before acid instillation, (4) rabbits (n=3) treated with 20 mg/kg IV 40 kD branched PEG-6G4V11N35A Fab' at 10 minutes before acid instillation, and (5) rabbits (n=29) treated with 5 ml IV saline at 10 minutes before and 6 hours after acid instillation.

5 Fig. 70D is a graph depicting total leukocyte (WBC) count in BAL fluid (represented in millions of cells counted in 20 ml BAL fluid) obtained at 72 hours post reperfusion for: (1) rabbits (n=5) treated with 7 mg/kg IV 20 kD linear PEG-6G4V11N35E Fab' at 10 minutes before and 6 hours after acid instillation, (2) rabbits (n=7) treated with 5 mg/kg IV full length IgG murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 at 10 minutes before acid instillation, 10 (3) rabbits (n=3) treated with 5 mg/kg IV 40 kD branched PEG-6G4V11N35A Fab' at 10 minutes before acid instillation, (4) rabbits (n=3) treated with 20 mg/kg IV 40 kD branched PEG-6G4V11N35A Fab' at 10 minutes before acid instillation, and (5) rabbits (n=11) treated with 5 ml IV saline at 10 minutes before and 6 hours after acid instillation.

15 Fig. 70E is a graph depicting total polymorphonuclear (PMN) cell count in BAL fluid (represented in millions of cells counted in 20 ml BAL fluid) obtained at 72 hours post reperfusion for: (1) rabbits (n=5) treated with 7 mg/kg IV 20 kD linear PEG-6G4V11N35E Fab' at 10 minutes before and 6 hours after acid instillation, (2) rabbits (n=7) treated with 5 mg/kg IV full length IgG murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 at 10 minutes before acid instillation, (3) rabbits (n=3) treated with 5 mg/kg IV 40 kD branched PEG-6G4V11N35A Fab' at 10 minutes before acid instillation, (4) rabbits (n=3) treated with 20 mg/kg IV 40 kD branched PEG-6G4V11N35A Fab' at 10 minutes before acid instillation, and (5) rabbits (n=9) treated with 5 ml IV saline at 10 minutes before and 6 hours after acid instillation. 20

25 Fig. 71 is a graph depicting the effect of pegylated anti-IL-8 Fab' (as measured by percent change in ear volume at 1, 2 and 3 days post reperfusion) in a rabbit ear model of ischemia reperfusion injury. The data points from animals treated with empty vehicle (n=11), full length IgG murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 (n=4), 20 kD linear PEG-6G4V11N35E Fab' (n=3), 30 kD linear PEG-6G4V11N35E Fab' (n=3), and 40 kD branched PEG-6G4V11N35E Fab' (n=3) are denoted by open boxes, open diamonds, open circles, open

triangles, and crossed boxes, respectively.

Fig. 72 is a graph comparing the serum concentration vs. time profiles of 20 kD linear PEG-maleimide modified Y0317 anti-human VEGF Fab' (denoted as "20K rhuMAb VEGF Fab IV") and 40 kD branched PEG-maleimide modified Y0317 anti-human VEGF Fab' (denoted as "40K rhuMAb VEGF Fab IV") molecules administered intravenously in mice.

Fig. 73 is a graph comparing the serum concentration vs. time profiles of 20 kD linear PEG-maleimide modified Y0317 anti-human VEGF Fab' (denoted as "20K rhuMAb VEGF Fab IP") and 40 kD branched PEG-maleimide modified Y0317 anti-human VEGF Fab' (denoted as "40K rhuMAb VEGF Fab IP") molecules administered intraperitoneally in mice.

Fig. 74 is a graph comparing inhibition of tumor growth in vivo in mice by intraperitoneal administration of 40 kD branched PEG-maleimide modified Y0317 anti-human VEGF Fab' (2 mg/kg loading dose on day 1 followed by 0.9 mg/kg/day maintenance dose for the remainder of the study) (denoted "40K-LOW"), 40 kD branched PEG-maleimide modified Y0317 anti-human VEGF Fab' (6 mg/kg loading dose on day 1 followed by 2.7 mg/kg/day maintenance dose for the remainder of the study) (denoted "40K-HIGH"), 40 kD branched PEG-6G4V11N35E Fab' (6 mg/kg loading dose on day 1 followed by 2.7 mg/kg/day maintenance dose for the remainder of the study) (denoted "CNTRL FAB"), Y0317 anti-human VEGF MAb (8 mg/kg loading dose on day 1 followed by 0.8 mg/kg maintenance dose every third day for the remainder of the study) (denoted "2ND GEN AB"), and phosphate buffered saline at physiological pH (0.1 ml/day for the duration of the study) (denoted "PBS").

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

### I. DEFINITIONS

In general, the following words or phrases have the indicated definition when used in the description, examples, and claims.

"Polymerase chain reaction" or "PCR" refers to a procedure or technique in which minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195 issued 28 July 1987. Generally, sequence information from the ends of the

region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers can coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. See generally Mullis *et al.*, Cold Spring Harbor Symp. Quant. Biol. 51:263 (1987); Erlich, ed., PCR Technology (Stockton Press, NY, 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample comprising the use of a known nucleic acid as a primer and a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies and immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain ( $V_H$ ) followed by a number of constant domains. Each light chain has a variable domain at one end ( $V_L$ ) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains (Clothia *et al.*, J. Mol. Biol. 186:651 (1985); Novotny and Haber, Proc. Natl. Acad. Sci. U.S.A. 82:4592 (1985)).

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a  $\beta$ -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the  $\beta$ -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, Sequences of Proteins of Immunological Interest, Fifth Edition, National Institute of Health, Bethesda, MD (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an  $F(ab')_2$  fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. In a two-chain Fv species, this region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv species (scFv), one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an

antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site. For a review of scFv see Pluckthun, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

5 The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')<sub>2</sub> antibody  
10 fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

15 Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these can be further divided into subclasses (isotypes), e.g., IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, IgG<sub>4</sub>, IgA<sub>1</sub>, and IgA<sub>2</sub>. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ,  
20 respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist and antagonist antibodies) and antibody compositions with polyepitopic specificity.

25 "Antibody fragment", and all grammatical variants thereof, as used herein are defined as a portion of an intact antibody comprising the antigen binding site or variable region of the intact antibody, wherein the portion is free of the constant heavy chain domains (i.e. CH2, CH3, and CH4, depending on antibody isotype) of the Fc region of the intact antibody. Examples of

antibody fragments include Fab, Fab', Fab'-SH, F(ab')<sub>2</sub>, and Fv fragments; diabodies; any antibody fragment that is a polypeptide having a primary structure consisting of one uninterrupted sequence of contiguous amino acid residues (referred to herein as a "single-chain antibody fragment" or "single chain polypeptide"), including without limitation (1) single-chain Fv (scFv) molecules (2) single chain polypeptides containing only one light chain variable domain, or a fragment thereof that contains the three CDRs of the light chain variable domain, without an associated heavy chain moiety and (3) single chain polypeptides containing only one heavy chain variable region, or a fragment thereof containing the three CDRs of the heavy chain variable region, without an associated light chain moiety; and multispecific or multivalent structures formed from antibody fragments. In an antibody fragment comprising one or more heavy chains, the heavy chain(s) can contain any constant domain sequence (e.g. CH1 in the IgG isotype) found in a non-Fc region of an intact antibody, and/or can contain any hinge region sequence found in an intact antibody, and/or can contain a leucine zipper sequence fused to or situated in the hinge region sequence or the constant domain sequence of the heavy chain(s). Suitable leucine zipper sequences include the jun and fos leucine zippers taught by Kostelney et al., J. Immunol., 148: 1547-1553 (1992) and the GCN4 leucine zipper described in the Examples below.

Unless specifically indicated to the contrary, the term "conjugate" as described and claimed herein is defined as a heterogeneous molecule formed by the covalent attachment of one or more antibody fragment(s) to one or more polymer molecule(s), wherein the heterogeneous molecule is water soluble, i.e. soluble in physiological fluids such as blood, and wherein the heterogeneous molecule is free of any structured aggregate. In the context of the foregoing definition, the term "structured aggregate" refers to (1) any aggregate of molecules in aqueous solution having a spheroid or spheroid shell structure, such that the heterogeneous molecule is not in a micelle or other emulsion structure, and is not anchored to a lipid bilayer, vesicle or liposome; and (2) any aggregate of molecules in solid or insolubilized form, such as a chromatography bead matrix, that does not release the heterogeneous molecule into solution upon contact with an aqueous phase. Accordingly, the term "conjugate" as defined herein

encompasses the aforementioned heterogeneous molecule in a precipitate, sediment, bioerodible matrix or other solid capable of releasing the heterogeneous molecule into aqueous solution upon hydration of the solid.

Unless specifically indicated to the contrary, the terms "polymer", "polymer molecule", "nonproteinaceous polymer", and "nonproteinaceous polymer molecule" are used interchangeably and are defined as a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is contained in the group consisting of alanine (Ala), cysteine (Cys), aspartic acid (Asp), glutamic acid (Glu), phenylalanine (Phe), glycine (Gly), histidine (His), isoleucine (Ile), lysine (Lys), leucine (Leu), methionine (Met), asparagine (Asn), proline (Pro), glutamine (Gln), arginine (Arg), serine (Ser), threonine (Thr), valine (Val), tryptophan (Trp), and tyrosine (Tyr) residues.

The term "monoclonal antibody" (mAb) as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each mAb is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they can be synthesized by hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567 to Cabilly *et al.*). The "monoclonal antibodies" also include clones of antigen-recognition and binding-site containing antibody fragments (Fv clones) isolated from phage antibody libraries using the techniques described in Clackson *et al.*, *Nature*, 352:624-628 (1991)



and Marks *et al.*, *J. Mol. Biol.*, **222**:581-597 (1991), for example.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-IL-8 antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. (See, e.g., U.S. Pat. No. 4,816,567 to Cabilly *et al.*; Mage and Lamoyi, in Monoclonal Antibody Production Techniques and Applications, pp. 79-97 (Marcel Dekker, Inc., New York, 1987).)

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (Cabilly *et al.*, *supra*; Morrison *et al.*, Proc. Natl. Acad. Sci. U.S.A. 81:6851 (1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub>, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies can comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance.

In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see Jones *et al.*, Nature 321:522 (1986); Reichmann *et al.*, Nature 332:323 (1988); and Presta, Curr. Op. Struct. Biol. 2:593 (1992).

The terms “human vascular endothelial growth factor”, “vascular endothelial growth factor”, “human VEGF” and “VEGF” are used interchangeably herein to refer to the 165-amino acid human vascular endothelial cell growth factor polypeptide, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factor polypeptides, described by Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991), together with the naturally occurring allelic and processed forms of such growth factor polypeptides.

The term “human VEGF receptor”, “VEGF receptor”, “human VEGFr” and “VEGFr” are used interchangeably herein to refer to a cellular receptor for VEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof which retain the ability to bind human VEGF. One example of a VEGF receptor is the *fms*-like tyrosine kinase (*flt*), a transmembrane receptor in the tyrosine kinase family. DeVries *et al.*, *Science* 255:989 (1992); Shibuya *et al.*, *Oncogene* 5:519 (1990). The *flt* receptor comprises an extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of VEGF, whereas the intracellular domain is involved in signal transduction. Another example of a VEGF receptor is the *flk-1* receptor (also referred to as KDR). Matthews *et al.*, *Proc. Nat. Acad. Sci.* 88:9026 (1991); Terman *et al.*, *Oncogene* 6:1677 (1991); Terman *et al.*, *Biochem. Biophys. Res. Commun.* 187:1579 (1992). Binding of VEGF to the *flt* receptor results in the formation of at least two high molecular weight complexes, having apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of VEGF.

As used herein, the terms “human p185 receptor-like tyrosine kinase”, “c-Erb-B2”, “ErbB2”, “HER2”, and “HER2 receptor” are used interchangeably to refer to the c-Erb-B2 polypeptide described in Yamamoto et al., Nature, 319: 230-234 (1986) (Genebank accession number X03363).

5 As used herein, the terms “human CD20” and “CD20” refer to the B1 cell-surface antigen (CD20) polypeptide described in Tedder et al., Proc. Natl. Acad. Sci. (USA), 85: 208-212 (1988).

As used herein, the terms “human CD18” and “CD18” refer to the integrin  $\beta$ -chain polypeptide (CD18) described in Kishimoto et al., Cell, 48: 681-690 (1987).

10 As used herein, the terms “human CD11a” and “CD11a” refer to the human CD11a polypeptide described in Edwards et al., J. Biol. Chem., 270: 12635-12640 (1995), van Kooyk et al., J. Exp. Med., 183(3): 1247-1252 (1996), or Champe et al., J. Biol. Chem., 270: 1388-1394 (1995).

As used herein, the terms “human IgE” and “IgE” refer to any human immunoglobulin of the E isotype or class that binds to the human Fc $\epsilon$ RI receptor  $\alpha$ -chain.

15 As used herein, the terms “human Fc $\epsilon$ RI receptor  $\alpha$ -chain”, “Fc $\epsilon$ RI receptor  $\alpha$ -chain”, “human Fc $\epsilon$ RI receptor”, “Fc $\epsilon$ RI receptor”, “human Fc $\epsilon$ RI”, and “Fc $\epsilon$ RI” are used interchangeably to refer to the human Fc $\epsilon$ RI  $\alpha$ -chain polypeptide described by Shimizu et al., Proc. Natl. Acad. Sci. (USA), 85: 1907-1911 (1988).

20 As used herein, the terms “human Apo-2 receptor”, “Apo-2 receptor”, “human Apo-2”, and “Apo-2” are used interchangeably to refer to the Apo-2 polypeptide described in Fig. 1 of WO 98/51793 (published November 19, 1998) (International Application No. PCT/US98/09704).

25 As used herein, the terms “human tumor necrosis factor- $\alpha$ ”, “tumor necrosis factor- $\alpha$ ”, “human TNF- $\alpha$ ”, and “TNF- $\alpha$ ” are used interchangeably to refer to the human TNF- $\alpha$  polypeptide described in Pennica et al., Nature, 512: 721 (1984) or in Fig. 10 of U.S. Pat. No. 4,650,674.

As used herein, the terms “human tissue factor” and “tissue factor” are used to refer to the human tissue factor polypeptide described in Fig. 2 of European Patent No. 0 278 776 B1

(granted May 28, 1997).

As used herein, the terms “human  $\alpha_4$  integrin”, “ $\alpha_4$  integrin”, “human  $\alpha_4$ ”, and “ $\alpha_4$ ” are used interchangeably to refer to the human VLA-4  $\alpha_4$  subunit polypeptide described in Takada et al., EMBO J., 8: 1361-1368 (1989).

As used herein, the terms “human  $\beta_7$  integrin”, “ $\beta_7$  integrin”, “human  $\beta_7$ ”, and “ $\beta_7$ ” are used interchangeably to refer to the  $\beta_7$ -related integrin polypeptide described in Yuan et al., International Immunology, 2: 1097-1108 (1990).

As used herein, the terms “human GPIIIa integrin”, “GPIIIa integrin”, “human GPIIIa”, and “GPIIIa” are used interchangeably to refer to the GPIIIa polypeptide described in Fitzgerald et al., J. Biol. Chem., 262(9): 3936 (1987).

As used herein, the terms “human GPIIb integrin”, “GPIIb integrin”, “human GPIIb” and “GPIIb” are used interchangeably to refer to the GPIIb polypeptide described in Fitzgerald et al., Biochem., 26: 8158 (1987).

As used herein, the terms “human GPIIb-IIIa integrin”, “GPIIb-IIIa integrin”, “human GPIIb-IIIa”, and “GPIIb-IIIa” are used interchangeably to refer to a GPIIb-GPIIIa integrin complex.

As used herein, the terms “human epidermal growth factor receptor”, “epidermal growth factor receptor”, “human EGFR”, and “EGFR” are used interchangeably to refer to the human epidermal growth factor receptor polypeptide described in Ullrich et al., Nature, 309: 418-425 (1984).

As used herein, the terms “human CD3” and “CD3” are used interchangeably to refer to the 20K T3 glycoprotein subunit of the human T-cell receptor complex described in van den Elsen et al., Nature, 312: 413-418 (1984).

As used herein, the terms “human interleukin-2 receptor  $\alpha$ -chain”, “interleukin-2 receptor  $\alpha$ -chain”, “human IL-2R  $\alpha$ -chain”, “IL-2R  $\alpha$ -chain”, “human T-cell activation antigen”, “human TAC”, and “TAC” are used interchangeably to refer to the 272 amino acid interleukin-2 receptor polypeptide described in Leonard et al., Nature, 311: 626-631 (1984).

As used herein, the terms "anti-LFA-1 antibody", "anti-LFA-1 monoclonal antibody" and "anti-LFA-1 MAb" refer to an antibody directed against either CD11a or CD18 or both. The anti-CD11a antibodies include, e.g., MHM24 [Hildreth *et al.*, Eur. J. Immunol., 13: 202-208 (1983)], R3.1 (IgG1) [R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT], 25-3 (or 25.3), an IgG1 available from Immunotech, France [Olive *et al.*, in Feldmann, ed., Human T cell Clones. A new Approach to Immune Regulation, Clifton, NJ, Humana, 1986 p. 173], KBA (IgG2a) [Nishimura *et al.*, Cell. Immunol., 107: 32 (1987); Nishimura *et al.*, *ibid.*, 94: 122 (1985)], M7/15 (IgG2b) [Springer *et al.*, Immunol. Rev., 68: 171 (1982)], IOT16 [Vermot Desroches *et al.*, Scand. J. Immunol., 33: 277-286 (1991)], SPVL7 [Vermot Desroches *et al.*, *supra*], and M17 (IgG2a), available from ATCC, which are rat anti-murine CD11a antibodies.

Examples of anti-CD18 antibodies include MHM23 [Hildreth *et al.*, *supra*], M18/2 (IgG2a) [Sanches-Madrid *et al.*, J. Exp. Med., 158: 586 (1983)], H52 [Fekete *et al.*, J. Clin. Lab Immunol., 31: 145-149 (1990)], Mas191c [Vermot Desroches *et al.*, *supra*], IOT18 [Vermot Desroches *et al.*, *supra*], 60.3 [Taylor *et al.*, Clin. Exp. Immunol., 71: 324-328 (1988)], and 60.1 [Campana *et al.*, Eur. J. Immunol., 16: 537-542 (1986)].

The term "graft" as used herein refers to biological material derived from a donor for transplantation into a recipient. Grafts include such diverse material as, for example, isolated cells such as islet cells, tissue such as the amniotic membrane of a newborn, bone marrow, hematopoietic precursor cells, and organs such as skin, heart, liver, spleen, pancreas, thyroid lobe, lung, kidney, tubular organs (e.g., intestine, blood vessels, or esophagus), etc. The tubular organs can be used to replace damaged portions of esophagus, blood vessels, or bile duct. The skin grafts can be used not only for burns, but also as a dressing to damaged intestine or to close certain defects such as diaphragmatic hernia. The graft is derived from any mammalian source, including human, whether from cadavers or living donors. Preferably the graft is bone marrow or an organ such as heart and the donor of the graft and the host are matched for HLA class II antigens.

The term "donor" as used herein refers to the mammalian species, dead or alive, from which the graft is derived. Preferably, the donor is human. Human donors are preferably volunteer blood-related donors that are normal on physical examination and of the same major ABO blood group, because crossing major blood group barriers possibly prejudices survival of the allograft. It is, however, possible to transplant, for example, a kidney of a type O donor into an A, B or AB recipient.

The term "transplant" and variations thereof refers to the insertion of a graft into a host, whether the transplantation is syngeneic (where the donor and recipient are genetically identical), allogeneic (where the donor and recipient are of different genetic origins but of the same species), or xenogeneic (where the donor and recipient are from different species). Thus, in a typical scenario, the host is human and the graft is an isograft, derived from a human of the same or different genetic origins. In another scenario, the graft is derived from a species different from that into which it is transplanted, such as a baboon heart transplanted into a human recipient host, and including animals from phylogenically widely separated species, for example, a pig heart valve, or animal beta islet cells or neuronal cells transplanted into a human host.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal herein is human.

As used herein, protein, peptide and polypeptide are used interchangeably to denote an amino acid polymer or a set of two or more interacting or bound amino acid polymers.

As used herein, the term "inflammatory disorders" refers to pathological states resulting in inflammation, typically caused by neutrophil chemotaxis. Examples of such disorders include inflammatory skin diseases including psoriasis and atopic dermatitis; systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); ischemic reperfusion disorders including surgical tissue reperfusion injury,

myocardial ischemic conditions such as myocardial infarction, cardiac arrest, reperfusion after cardiac surgery and constriction after percutaneous transluminal coronary angioplasty, stroke, and abdominal aortic aneurysms; cerebral edema secondary to stroke; cranial trauma; hypovolemic shock; asphyxia; adult respiratory distress syndrome; acute lung injury; Behcet's Disease; dermatomyositis; polymyositis; multiple sclerosis; dermatitis; meningitis; encephalitis; uveitis; osteoarthritis; lupus nephritis; autoimmune diseases such as rheumatoid arthritis, Sjorgen's syndrome, vasculitis; diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder, multiple organ injury syndrome secondary to septicaemia or trauma; alcoholic hepatitis; bacterial pneumonia; antigen-antibody complex mediated diseases including glomerulonephritis; sepsis; sarcoidosis; immunopathologic responses to tissue/organ transplantation; inflammations of the lung, including pleurisy, alveolitis, vasculitis, pneumonia, chronic bronchitis, bronchiectasis, diffuse panbronchiolitis, hypersensitivity pneumonitis, idiopathic pulmonary fibrosis (IPF), and cystic fibrosis; etc. The preferred indications include acute lung injury, adult respiratory distress syndrome, ischemic reperfusion (including surgical tissue reperfusion injury, myocardial ischemia, and acute myocardial infarction), hypovolemic shock, asthma, bacterial pneumonia and inflammatory bowel disease such as ulcerative colitis.

As used herein, the term "LFA-1-mediated disorder" refers to pathological states caused by cell adherence interactions involving the LFA-1 receptor on lymphocytes. Examples of such disorders include T cell inflammatory responses such as inflammatory skin diseases including psoriasis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); adult respiratory distress syndrome; dermatitis; meningitis; encephalitis; uveitis; allergic conditions such as eczema and asthma and other conditions involving infiltration of T cells and chronic inflammatory responses; skin hypersensitivity reactions (including poison ivy and poison oak); atherosclerosis; leukocyte adhesion deficiency; autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE), diabetes mellitus, multiple sclerosis, Reynaud's syndrome, autoimmune thyroiditis, experimental autoimmune encephalomyelitis, Sjorgen's syndrome, juvenile onset diabetes, and immune responses associated with delayed hypersensitivity mediated by cytokines and T-lymphocytes typically

found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis; pernicious anemia; diseases involving leukocyte diapedesis; CNS inflammatory disorder, multiple organ injury syndrome secondary to septicemia or trauma; autoimmune haemolytic anemia; myethemia gravis; antigen-antibody complex mediated diseases; all types of transplantations, including graft vs. host or host vs. graft disease; etc.

As used herein, the term "IgE-mediated disorder" means a condition or disease which is characterized by the overproduction and/or hypersensitivity to the immunoglobulin IgE. Specifically it should be construed to include conditions associated with anaphylactic hypersensitivity and atopic allergies, including for example: asthma, allergic rhinitis & conjunctivitis (hay fever), eczema, urticaria and food allergies. However, the serious physiological condition of anaphylactic shock, usually caused by bee or snake stings or parental medication is also encompassed under the scope of this term.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The terms "allergy" and "atopy" and all their grammatical variants are used synonymously herein to refer to any disease mediated by a Type I (Gell & Coombs classification) hypersensitivity reaction, including allergic rhinitis, atopic dermatitis, anaphylaxis, allergic asthma.

As used herein, the terms "asthma", "asthmatic disorder", "asthmatic disease", and "bronchial asthma" refer to a condition of the lungs in which there is widespread narrowing of



lower airways. "Atopic asthma" and "allergic asthma" refer to asthma that is a manifestation of an IgE-mediated hypersensitivity reaction in the lower airways, including, e.g., moderate or severe chronic asthma, such as conditions requiring the frequent or constant use of inhaled or systemic steroids to control the asthma symptoms. A preferred indication is allergic asthma.

5 The term "allergic rhinitis" as used herein refers to any allergen-induced nasal symptoms, including itching, sneezing, nasal congestion, nasal discharge, and symptoms associated with nasal mucosal inflammation.

The terms "thrombotic disorder" and "prothrombotic disorder" as used interchangeably herein to refer to pathological conditions in which the blood coagulation cascade is activated (see, generally, Hoffbrand & Pettit, Essential Haematology, Blackwell Scientific Publications, 10 Oxford (1980)). Such conditions include peripheral arterial obstruction, acute myocardial infarction, deep vein thrombosis, pulmonary embolism, dissecting aneurysm, transient ischemic attack, restenosis, stroke and other occlusive disease or disorders such as unstable angina, disseminated intravascular coagulation, sepsis, surgical or infective shock, postoperative and 15 post-delivery trauma, angioplasty, cardiopulmonary bypass and coronary bypass, incompatible blood transfusion, amotio placentae, thrombotic thrombocytopenic purpura, asthma, chronic or acute renal disease, diabetes, inflammations, atherosclerosis, hemolytic uremic syndrome, symmetric peripheral necrosis, and allograft rejection in mammals including human.

The terms "hydrodynamic size", "apparent size", "apparent molecular weight", "effective size" and "effective molecular weight" of a molecule are used synonymously herein refer to the size of a molecule as determined by comparison to a standard curve produced with globular protein molecular weight standards in a size exclusion chromatography system, wherein the standard curve is created by mapping the actual molecular weight of each standard against its elution time observed in the size exclusion chromatography system. Thus, the apparent size of a 20 test molecule is derived by using the molecule's elution time to extrapolate a putative molecular weight from the standard curve. Preferably, the molecular weight standards used to create the standard curve are selected such that the apparent size of the test molecule falls within the linear portion of the standard curve.

## II. MODES FOR CARRYING OUT THE INVENTION

In one part, the invention arises from the surprising and unexpected discovery that antibody fragment-polymer conjugates having an effective or apparent size significantly greater than the antibody fragment-polymer conjugates described in the art confers an increase in serum half-life, an increase in mean residence time in circulation (MRT), and/or a decrease in serum clearance rate over underivatized antibody fragment which far exceed the modest changes in such biological property or properties obtained with the art-known antibody fragment-polymer conjugates. The present inventors have determined for the first time that increasing the effective size of an antibody fragment to at least about 500,000 D, or increasing the effective size of an antibody fragment by at least about 8 fold over the effective size of the parental antibody fragment, or derivatizing an antibody fragment with a polymer of at least about 20,000 D in molecular weight, yields a molecule with a commercially useful pharmacokinetic profile. The greatly extended serum half-life, extended MRT, and/or reduced serum clearance rate of the conjugates of the invention makes such conjugates viable alternatives to intact antibodies used for therapeutic treatment of many disease indications. Antibody fragments provide significant advantages over intact antibodies, notably the fact that recombinant antibody fragments can be made in bacterial cell expression systems. Bacterial cell expression systems provide several advantages over mammalian cell expression systems, including reduced time and cost at both the research and development and manufacturing stages of a product.

In another part, the present invention also arises from the humanization of the 6G4.2.5 murine anti-rabbit IL-8 monoclonal antibody ("6G4.2.5") described in WO 95/23865 (PCT/US95/02589 published September 8, 1995), the entire disclosure of which is specifically incorporated herein by reference. The hybridoma producing antibody 6G4.2.5 was deposited on September 28, 1994 with the American Type Culture Collection and assigned ATCC Accession No. HB 11722 as described in the Examples below. In one aspect, the invention provides a humanized derivative of the 6G4.2.5 antibody, variant 11 (referred to herein as "6G4.2.5v11"), in which the murine CDRs of 6G4.2.5 are grafted onto a consensus framework for human light chain  $\kappa$ I and human IgG1 heavy chain subgroup III, followed by importing three framework

residues from the murine 6G4.2.5 parent heavy chain variable domain sequence into analogous sites in the heavy chain variable domain of the human template sequence, as described in the Examples below. In another aspect, the invention provides variants of the 6G4.2.5v11 antibody with certain amino acid substitution(s) yielding increased affinity for human IL-8 and/or promoting greater efficiency in recombinant manufacturing processes.

It will be understood that in the context of this Section (II) and all subsections thereof, every reference to "an antibody fragment" or "the antibody fragment" contained in a conjugate shall be a reference to one or more antibody fragment(s) in the conjugate (consistent with the definition of the term "conjugate" set forth in Section (I) above), except where the number of antibody fragment(s) in the conjugate is expressly indicated. It will be understood that in the context of this Section (II) and all subsections thereof, every reference to "a polymer", "a polymer molecule", "the polymer", or "the polymer molecule" contained in a conjugate shall be a reference to one or more polymer molecule(s) in the conjugate (consistent with the definition of the term "conjugate" set forth in Section (I) above), except where the number of polymer molecule(s) in the conjugate is expressly indicated.

#### 1. LARGE EFFECTIVE SIZE ANTIBODY FRAGMENT-POLYMER CONJUGATES

In one aspect, the invention provides an antibody fragment covalently attached to a polymer to form a conjugate having an effective or apparent size of at least about 500,000 Daltons (D). In another aspect, the invention provides an antibody fragment covalently attached to a polymer to form a conjugate having an apparent size that is at least about 8 fold greater than the apparent size of the parental antibody fragment. In yet another aspect, the invention provides an antibody fragment covalently attached to a polymer of at least about 20,000 D in molecular weight (MW). It will be appreciated that the unexpectedly and surprisingly large increase in antibody fragment serum half-life, increase in MRT, and/or decrease in serum clearance rate can be achieved by using any type of polymer or number of polymer molecules which will provide the conjugate with an effective size of at least about 500,000 D, or by using any type of polymer or number of polymer molecules which will provide the conjugate with an effective size that is at least about 8 fold greater than the effective size of the parental antibody fragment, or by using

any type or number of polymers wherein each polymer molecule is at least about 20,000 D in MW. Thus, the invention is not dependent on the use of any particular polymer or molar ratio of polymer to antibody fragment in the conjugate.

In addition, the beneficial aspects of the invention extend to antibody fragments without regard to antigen specificity. Although variations from antibody to antibody are to be expected, the antigen specificity of a given antibody will not substantially impair the extraordinary improvement in serum half-life, MRT, and/or serum clearance rate for antibody fragments thereof that can be obtained by derivatizing the antibody fragments as taught herein. The invention can be applied to an antibody fragment specific for any antigen of interest, including, e.g., renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor (TF), and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF- $\beta$ ; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, TGF- $\beta$ 4, or TGF- $\beta$ 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I

(brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), *e.g.*, M-CSF, GM-CSF, and G-CSF; interleukins (ILs), *e.g.*, IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the HIV envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

In one embodiment, the conjugate has an effective size of at least about 500,000 D, or at least about 800,000 D, or at least about 900,000 D, or at least about 1,000,000 D, or at least about 1,200,000 D, or at least about 1,400,000 D, or at least about 1,500,000 D, or at least about 1,800,000 D, or at least about 2,000,000 D, or at least about 2,500,000 D.

In another embodiment, the conjugate has an effective size of at or about 500,000 D to at or about 10,000,000 D, or an effective size of at or about 500,000 D to at or about 8,000,000 D, or an effective size of at or about 500,000 D to at or about 5,000,000 D, or an effective size of at or about 500,000 D to at or about 4,000,000 D, or an effective size of at or about 500,000 D to at or about 3,000,000 D, or an effective size of at or about 500,000 D to at or about 2,500,000 D, or an effective size of at or about 500,000 D to at or about 2,000,000 D, or an effective size of at or about 500,000 D to at or about 1,800,000 D, or an effective size of at or about 500,000 D to at or about 1,600,000 D, or an effective size of at or about 500,000 D to at or about 1,500,000 D, or an effective size of at or about 500,000 D to at or about 1,000,000 D.

In another embodiment, the conjugate has an effective size of at or about 800,000 D to at or about 10,000,000 D, or an effective size of at or about 800,000 D to at or about 8,000,000 D, or an effective size of at or about 800,000 D to at or about 5,000,000 D, or an effective size of at or about 800,000 D to at or about 4,000,000 D, or an effective size of at or about 800,000 D to at or about 3,000,000 D, or an effective size of at or about 800,000 D to at or about 2,500,000 D, or an effective size of at or about 800,000 D to at or about 2,000,000 D, or an effective size of at or

about 800,000 D to at or about 1,800,000 D, or an effective size of at or about 800,000 D to at or about 1,600,000 D, or an effective size of at or about 800,000 D to at or about 1,500,000 D, or an effective size of at or about 800,000 D to at or about 1,000,000 D.

In another embodiment, the conjugate has an effective size of at or about 900,000 D to at or about 10,000,000 D, or an effective size of at or about 900,000 D to at or about 8,000,000 D, or an effective size of at or about 900,000 D to at or about 5,000,000 D, or an effective size of at or about 900,000 D to at or about 4,000,000 D, or an effective size of at or about 900,000 D to at or about 3,000,000 D, or an effective size of at or about 900,000 D to at or about 2,500,000 D, or an effective size of at or about 900,000 D to at or about 2,000,000 D, or an effective size of at or about 900,000 D to at or about 1,800,000 D, or an effective size of at or about 900,000 D to at or about 1,600,000 D, or an effective size of at or about 900,000 D to at or about 1,500,000 D.

In another embodiment, the conjugate has an effective size of at or about 1,000,000 D to at or about 10,000,000 D, or an effective size of at or about 1,000,000 D to at or about 8,000,000 D, or an effective size of at or about 1,000,000 D to at or about 5,000,000 D, or an effective size of at or about 1,000,000 D to at or about 4,000,000 D, or an effective size of at or about 1,000,000 D to at or about 3,000,000 D, or an effective size of at or about 1,000,000 D to at or about 2,500,000 D, or an effective size of at or about 1,000,000 D to at or about 2,000,000 D, or an effective size of at or about 1,000,000 D to at or about 1,800,000 D, or an effective size of at or about 1,000,000 D to at or about 1,600,000 D, or an effective size of at or about 1,000,000 D to at or about 1,500,000 D.

In a further embodiment, the conjugate has an effective size that is at least about 8 fold greater, or at least about 10 fold greater, or at least about 12 fold greater, or at least about 15 fold greater, or at least about 18 fold greater, or at least about 20 fold greater, or at least about 25 fold greater, or at least about 28 fold greater, or at least about 30 fold greater, or at least about 40 fold greater, than the effective size of the parental antibody fragment.

In another embodiment, the conjugate has an effective size that is about 8 fold to about 100 fold greater, or is about 8 fold to about 80 fold greater, or is about 8 fold to about 50 fold greater, or is about 8 fold to about 40 fold greater, or is about 8 fold to about 30 fold greater, or is

about 8 fold to about 28 fold greater, or is about 8 fold to about 25 fold greater, or is about 8 fold to about 20 fold greater, or is about 8 fold to about 18 fold greater, or is about 8 fold to about 15 fold greater, than the effective size of the parental antibody fragment.

In another embodiment, the conjugate has an effective size that is about 12 fold to about 100 fold greater, or is about 12 fold to about 80 fold greater, or is about 12 fold to about 50 fold greater, or is about 12 fold to about 40 fold greater, or is about 12 fold to about 30 fold greater, or is about 12 fold to about 28 fold greater, or is about 12 fold to about 25 fold greater, or is about 12 fold to about 20 fold greater, or is about 12 fold to about 18 fold greater, or is about 12 fold to about 15 fold greater, than the effective size of the parental antibody fragment.

In another embodiment, the conjugate has an effective size that is about 15 fold to about 100 fold greater, or is about 15 fold to about 80 fold greater, or is about 15 fold to about 50 fold greater, or is about 15 fold to about 40 fold greater, or is about 15 fold to about 30 fold greater, or is about 15 fold to about 28 fold greater, or is about 15 fold to about 25 fold greater, or is about 15 fold to about 20 fold greater, or is about 15 fold to about 18 fold greater, than the effective size of the parental antibody fragment.

In another embodiment, the conjugate has an effective size that is about 18 fold to about 100 fold greater, or is about 18 fold to about 80 fold greater, or is about 18 fold to about 50 fold greater, or is about 18 fold to about 40 fold greater, or is about 18 fold to about 30 fold greater, or is about 18 fold to about 28 fold greater, or is about 18 fold to about 25 fold greater, or is about 18 fold to about 20 fold greater, than the effective size of the parental antibody fragment.

In another embodiment, the conjugate has an effective size that is about 20 fold to about 100 fold greater, or is about 20 fold to about 80 fold greater, or is about 20 fold to about 50 fold greater, or is about 20 fold to about 40 fold greater, or is about 20 fold to about 30 fold greater, or is about 20 fold to about 28 fold greater, or is about 20 fold to about 25 fold greater, than the effective size of the parental antibody fragment.

In another embodiment, the conjugate has an effective size that is about 25 fold to about 100 fold greater, or is about 25 fold to about 80 fold greater, or is about 25 fold to about 50 fold greater, or is about 25 fold to about 40 fold greater, or is about 25 fold to about 30 fold greater, or

is about 25 fold to about 28 fold greater, than the effective size of the parental antibody fragment.

In another embodiment, the conjugate has an effective size that is about 28 fold to about 100 fold greater, or is about 28 fold to about 80 fold greater, or is about 28 fold to about 50 fold greater, or is about 28 fold to about 40 fold greater, or is about 28 fold to about 30 fold greater, than the effective size of the parental antibody fragment.

In another embodiment, the conjugate has an effective size that is about 30 fold to about 100 fold greater, or is about 30 fold to about 80 fold greater, or is about 30 fold to about 50 fold greater, or is about 30 fold to about 40 fold greater, than the effective size of the parental antibody fragment.

In another embodiment, the conjugate has an effective size that is about 40 fold to about 100 fold greater, or is about 40 fold to about 80 fold greater, or is about 40 fold to about 50 fold greater, than the effective size of the parental antibody fragment.

In still another embodiment, the conjugate is an antibody fragment covalently attached to at least one polymer having an actual MW of at least about 20,000 D.

In a further embodiment, the conjugate is an antibody fragment covalently attached to at least one polymer having an actual MW of at least about 30,000 D.

In yet another embodiment, the conjugate is an antibody fragment covalently attached to at least one polymer having an actual MW of at least about 40,000 D.

In another embodiment, the conjugate is an antibody fragment covalently attached to at least one polymer having an actual MW that is at or about 20,000 D to at or about 300,000 D, or is at or about 30,000 D to at or about 300,000 D, or is at or about 40,000 D to at or about 300,000 D.

In another embodiment, the conjugate is an antibody fragment covalently attached to at least one polymer having an actual MW that is at or about 20,000 D to at or about 100,000 D, or is at or about 30,000 D to at or about 100,000 D, or is at or about 40,000 D to at or about 100,000 D.

In another embodiment, the conjugate is an antibody fragment covalently attached to at least one polymer having an actual MW that is at or about 20,000 D to at or about 70,000 D, or is



at or about 30,000 D to at or about 70,000 D, or is at or about 40,000 D to at or about 70,000 D.

In another embodiment, the conjugate is an antibody fragment covalently attached to at least one polymer having an actual MW that is at or about 20,000 D to at or about 50,000 D, or is at or about 30,000 D to at or about 50,000 D, or is at or about 40,000 D to at or about 50,000 D.

5 In another embodiment, the conjugate is an antibody fragment covalently attached to at least one polymer having an actual MW that is at or about 20,000 D to at or about 40,000 D, or is at or about 30,000 D to at or about 40,000 D.

The conjugates of the invention can be made using any suitable technique now known or hereafter developed for derivatizing antibody fragments with polymers. It will be appreciated  
10 that the invention is not limited to conjugates utilizing any particular type of linkage between an antibody fragment and a polymer.

The conjugates of the invention include species wherein a polymer is covalently attached to a non-specific site or non-specific sites on the parental antibody fragment, i.e. polymer attachment is not targeted to a particular region or a particular amino acid residue in the parental  
15 antibody fragment. In such embodiments, the coupling chemistry can, for example, utilize the free epsilon amino groups of lysine residues in the parental antibody as attachment sites for the polymer, wherein such lysine residue amino groups are randomly derivatized with polymer.

In addition, the conjugates of the invention include species wherein a polymer is covalently attached to a specific site or specific sites on the parental antibody fragment, i.e.  
20 polymer attachment is targeted to a particular region or a particular amino acid residue or residues in the parental antibody fragment. In such embodiments, the coupling chemistry can, for example, utilize the free sulfhydryl group of a cysteine residue not in a disulfide bridge in the parental antibody fragment. In one embodiment, one or more cysteine residue(s) is (are) engineered into a selected site or sites in the parental antibody fragment for the purpose of  
25 providing a specific attachment site or sites for polymer. The polymer can be activated with any functional group that is capable of reacting specifically with the free sulfhydryl or thiol group(s) on the parental antibody, such as maleimide, sulfhydryl, thiol, triflate, tesylate, aziridine, oxirane, and 5-pyridyl functional groups. The polymer can be coupled to the parental antibody fragment

using any protocol suitable for the chemistry of the coupling system selected, such as the protocols and systems described in Section (II)(1)(b) or in Section (T) of the Examples below.

In another embodiment, polymer attachment is targeted to the hinge region of the parental antibody fragment. The location of the hinge region varies according to the isotype of the parental antibody. Typically, the hinge region of IgG, IgD and IgA isotype heavy chains is contained in a proline rich peptide sequence extending between the C<sub>H</sub>1 and C<sub>H</sub>2 domains. In a preferred embodiment, a cysteine residue or residues is (are) engineered into the hinge region of the parental antibody fragment in order to couple polymer specifically to a selected location in the hinge region.

In one aspect, the invention encompasses a conjugate having any molar ratio of polymer to antibody fragment that endows the conjugate with an apparent size in the desired range as taught herein. The apparent size of the conjugate will depend in part upon the size and shape of the polymer used, the size and shape of the antibody fragment used, the number of polymer molecules attached to the antibody fragment, and the location of such attachment site(s) on the antibody fragment. These parameters can easily be identified and maximized to obtain the a conjugate with the desired apparent size for any type of antibody fragment, polymer and linkage system.

In another aspect, the invention encompasses a conjugate with a polymer to antibody fragment molar ratio of no more than about 10:1, or no more than about 5:1, or no more than about 4:1, or no more than about 3:1, or no more than about 2:1, or no more than 1:1.

In yet another aspect, the invention encompasses a conjugate wherein the antibody fragment is attached to about 10 or fewer polymer molecules, each polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In another embodiment, the conjugate contains an antibody fragment attached to about 5 or fewer polymer molecules, each polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In still another embodiment, the conjugate contains an antibody fragment attached to about 4 or fewer polymer molecules, each polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000

D, or at least about 40,000 D. In a further embodiment, the conjugate contains an antibody fragment attached to about 3 or fewer polymer molecules, each polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In an additional embodiment, the conjugate contains an antibody fragment attached to about  
5 2 or fewer polymer molecules, each polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. Also provided herein is a conjugate containing an antibody fragment attached to a single polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D.

10 In still another aspect, the invention encompasses a conjugate wherein every polymer molecule in the conjugate has a molecular weight that is at or about 20,000 D to at or about 300,000 D, or is at or about 30,000 D to at or about 300,000 D, or is at or about 40,000 D to at or about 300,000 D, and wherein the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

15 In still another aspect, the invention encompasses a conjugate wherein every polymer molecule in the conjugate has a molecular weight that is at or about 20,000 D to at or about 100,000 D, or is at or about 30,000 D to at or about 100,000 D, or is at or about 40,000 D to at or about 100,000 D, and wherein the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

20 In still another aspect, the invention encompasses a conjugate wherein every polymer molecule in the conjugate has a molecular weight that is at or about 20,000 D to at or about 70,000 D, or is at or about 30,000 D to at or about 70,000 D, or is at or about 40,000 D to at or about 70,000 D, and wherein the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no  
25

more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

In still another aspect, the invention encompasses a conjugate wherein every polymer molecule in the conjugate has a molecular weight that is at or about 20,000 D to at or about 50,000 D, or is at or about 30,000 D to at or about 50,000 D, or is at or about 40,000 D to at or about 50,000 D, and wherein the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

In still another aspect, the invention encompasses a conjugate wherein every polymer molecule in the conjugate has a molecular weight that is at or about 20,000 D to at or about 40,000 D, or is at or about 30,000 D to at or about 40,000 D, and wherein the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

It is believed that the serum half-life, MRT and/or serum clearance rate of any antibody fragment can be greatly improved by derivatizing the antibody fragment with polymer as taught herein. In one embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH, Fv, scFv and F(ab')<sub>2</sub>.

In a preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein every polymer molecule in the conjugate is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, every polymer molecule in the conjugate molecule is attached to the hinge region of the antibody fragment, and the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

In yet another preferred embodiment, the conjugate contains a  $F(ab')_2$  antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide  
 5 bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In a further embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule and the polymer is coupled to a cysteine residue in the light or  
 10 heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In an additional embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, every polymer molecule in the conjugate is at least about 20,000 D in molecular weight, or at least about 30,000 in molecular weight, or at least about 40,000 D in molecular weight, every polymer molecule in the conjugate is attached to the hinge region of the antibody fragment, and the conjugate contains no more than about 10  
 15 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, every polymer molecule in the conjugate is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in  
 25 molecular weight, every polymer molecule in the conjugate is attached to the hinge region of the antibody fragment, and the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1

polymer molecule.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, every polymer molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, every polymer molecule in the conjugate is attached to the hinge region of the antibody fragment, and the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, every polymer molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, every polymer molecule in the conjugate is attached to the hinge region of the antibody fragment, and the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, every polymer molecule in the conjugate is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, every polymer molecule in the conjugate is attached to the hinge region of the antibody fragment, and the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, every polymer molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, every polymer molecule in the conjugate is attached to the hinge region of the antibody fragment, and the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

In a further embodiment, the conjugate contains a F(ab')<sub>2</sub> antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at least about 20,000 D in molecular weight, or at least about 30,000 D in molecular weight, or at least about 40,000 D in molecular weight, and wherein every polymer molecule in the conjugate is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another embodiment, the conjugate contains a F(ab')<sub>2</sub> antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight, and wherein every polymer molecule in the conjugate is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another embodiment, the conjugate contains a F(ab')<sub>2</sub> antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or

about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, and wherein every polymer molecule in the conjugate is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another embodiment, the conjugate contains a  $F(ab')_2$  antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, and wherein every polymer molecule in the conjugate is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another embodiment, the conjugate contains a  $F(ab')_2$  antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, and wherein every polymer molecule in the conjugate is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another embodiment, the conjugate contains a  $F(ab')_2$  antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, and wherein every polymer molecule in the conjugate is



attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

5           In yet another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at least about 20,000 D in molecular weight, or at least about 30,000 D in molecular weight, or at least about 40,000 D in molecular weight, wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

10           In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight, wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

20           In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy

chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no  
5 more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy  
10 chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no  
15 more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy  
20 chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no  
25 more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In still another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at least about 20,000 D in molecular weight, or at least about 30,000 D in molecular weight, or at least about 40,000 D in molecular weight, and wherein the polymer molecule is attached to the hinge region of the antibody fragment.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight, and wherein the polymer molecule is attached to the hinge region of the antibody fragment.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, and wherein the polymer molecule is attached to the hinge region of the antibody fragment.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, and wherein the polymer molecule is attached to the hinge region of the antibody fragment.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in

molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, and wherein the polymer molecule is attached to the hinge region of the antibody fragment.

In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, and wherein the polymer molecule is attached to the hinge region of the antibody fragment.

Although any type of polymer is contemplated for use in constructing the conjugates of the invention, including the polymers and chemical linkage systems described in Section (II)(1)(b) below, polyethylene glycol (PEG) polymers are preferred for use herein.

In one embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual MW of at least about 20,000 D.

In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual MW of at least about 30,000 D.

In yet another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual MW of at least about 40,000 D.

In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual MW that is at or about 20,000 D to at or about 300,000 D, or is at or about 30,000 D to at or about 300,000 D, or is at or about 40,000 D to at or about 300,000 D.

In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual MW that is at or about 20,000 D to at or about 100,000 D, or is at or about 30,000 D to at or about 100,000 D, or is at or about 40,000 D to at or about 100,000 D.

In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual MW that is at or about 20,000 D to at or about 70,000 D, or is at or about 30,000 D to at or about 70,000 D, or is at or about 40,000 D to at or about 70,000 D.

In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual MW that is at or about 20,000 D to at or about 50,000 D, or is at

or about 30,000 D to at or about 50,000 D, or is at or about 40,000 D to at or about 50,000 D.

In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual MW that is at or about 20,000 D to at or about 40,000 D, or is at or about 30,000 D to at or about 40,000 D.

5 In another aspect, the invention encompasses a conjugate with a PEG to antibody fragment molar ratio of no more than about 10:1, or no more than about 5:1, or no more than about 4:1, or no more than about 3:1, or no more than about 2:1, or no more than 1:1.

In yet another aspect, the invention encompasses a conjugate wherein the antibody fragment is attached to about 10 or fewer PEG molecules, each PEG molecule having a  
10 molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In another embodiment, the conjugate contains an antibody fragment attached to about 5 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In still another embodiment, the conjugate contains an antibody fragment attached to about 4 or fewer PEG molecules, each PEG molecule  
15 having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In a further embodiment, the conjugate contains an antibody fragment attached to about 3 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In an additional embodiment, the conjugate contains an antibody fragment attached to about 2 or fewer PEG molecules, each  
20 PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. Also provided herein is a conjugate containing an antibody fragment attached to a single PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D.

In another aspect, the invention encompasses a conjugate wherein the antibody fragment  
25 is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5

PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In another aspect, the invention encompasses a conjugate wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In another aspect, the invention encompasses a conjugate wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In another aspect, the invention encompasses a conjugate wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In another aspect, the invention encompasses a conjugate wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than

about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In still another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and F(ab')<sub>2</sub>, wherein the antibody fragment is attached to about 10 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In another embodiment, the foregoing conjugate contains an antibody fragment attached to about 5 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In still another embodiment, the foregoing conjugate contains an antibody fragment attached to about 4 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In a further embodiment, the foregoing conjugate contains an antibody fragment attached to about 3 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In an additional embodiment, the foregoing conjugate contains an antibody fragment attached to about 2 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. Also provided herein is the foregoing conjugate that contains an antibody fragment attached to a single PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D.

In another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and F(ab')<sub>2</sub>, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG

molecule.

In another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and F(ab')<sub>2</sub>, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and F(ab')<sub>2</sub>, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and F(ab')<sub>2</sub>, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.



In another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and F(ab')<sub>2</sub>, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In a preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG having a molecular weight of at least about 20,000D, or at least about 30,000D, or at least about 40,000D, and wherein every PEG molecule in the conjugate is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG having a molecular weight that is at or about 20,000 D to about 300,000 D, or is at or about 30,000 D to at or about 300,000 D, or is at or about 40,000 D to at or about 300,000 D, and wherein every PEG molecule in the conjugate is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG having a molecular weight that is at or about 20,000 D to about 100,000 D, or is at or about 30,000 D to at or about 100,000 D, or is at or about 40,000 D to at or about 100,000 D, and wherein every PEG molecule in the conjugate is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG having a molecular weight that is at or about 20,000 D to about 70,000 D,

or is at or about 30,000 D to at or about 70,000 D, or is at or about 40,000 D to at or about 70,000 D, and wherein every PEG molecule in the conjugate is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG having a molecular weight that is at or about 20,000 D to about 50,000 D, or is at or about 30,000 D to at or about 50,000 D, or is at or about 40,000 D to at or about 50,000 D, and wherein every PEG molecule in the conjugate is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG having a molecular weight that is at or about 20,000 D to about 40,000 D, or is at or about 30,000 D to at or about 40,000 D, and wherein every PEG molecule in the conjugate is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at least about 20,000D in molecular weight, or at least about 30,000D in molecular weight, or at least about 40,000D in molecular weight, wherein every PEG molecule in the conjugate molecule is attached to the hinge region of the antibody fragment, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight,

wherein every PEG molecule in the conjugate molecule is attached to the hinge region of the antibody fragment, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, wherein every PEG molecule in the conjugate molecule is attached to the hinge region of the antibody fragment, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, wherein every PEG molecule in the conjugate molecule is attached to the hinge region of the antibody fragment, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at

or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, wherein every PEG molecule in the conjugate molecule is attached to the hinge region of the antibody fragment, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, wherein every PEG molecule in the conjugate molecule is attached to the hinge region of the antibody fragment, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

In yet another preferred embodiment, the conjugate contains a  $F(ab')_2$  antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at least about 20,000D in molecular weight, or at least about 30,000D in molecular weight, or at least about 40,000D in molecular weight, wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein every PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another preferred embodiment, the conjugate contains a  $F(ab')_2$  antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight,

wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein every PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another preferred embodiment, the conjugate contains a  $F(ab')_2$  antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein every PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another preferred embodiment, the conjugate contains a  $F(ab')_2$  antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein every PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another preferred embodiment, the conjugate contains a  $F(ab')_2$  antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein

every PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

5 In another preferred embodiment, the conjugate contains a  $F(ab')_2$  antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein every PEG molecule is attached to a cysteine residue in the light or  
10 heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In still another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at least about 20,000 D in  
15 molecular weight, or at least about 30,000 in molecular weight, or at least about 40,000 D in molecular weight, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy  
20 chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at  
25 or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody

fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight,

wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

It will be appreciated that all of the above-described embodiments of the invention utilizing PEG polymers include conjugates wherein the PEG polymer(s) is (are) linear or branched. In a preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and at least about 40,000 D in molecular weight. In a particularly surprising and unexpected finding, the inventors discovered that the foregoing conjugate exhibits a serum half-life, MRT and serum clearance rate approaching that of full length antibody as shown in Example X below.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 40,000 D to at or about 300,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached



to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 40,000 D to at or about 100,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 40,000 D to at or about 70,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 40,000 D to at or about 50,000 D.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and at least 40,000D in molecular weight, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 40,000 D to at or about 300,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 40,000 D to at or about 100,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is

branched and has a molecular weight that is at or about 40,000 D to at or about 70,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 40,000 D to at or about 50,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In a preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and at least about 40,000 D in molecular weight.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 40,000 D to at or about 300,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 40,000 D to at or about 100,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 40,000 D to at or about 70,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 40,000 D to at or about 50,000 D.

In another preferred embodiment, the invention provides a conjugate containing an

antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and at least 40,000D in molecular weight, and the PEG molecule is attached to the hinge region of the antibody fragment.

5 In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 40,000 D to at or about 300,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

10 In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 40,000 D to at or about 100,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

15 In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 40,000 D to at or about 70,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

20 In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 40,000 D to at or about 50,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

25 In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at least about 30,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 30,000 D to at or about 300,000 D.

5 In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 30,000 D to at or about 100,000 D.

10 In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 30,000 D to at or about 70,000 D.

15 In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 30,000 D to at or about 50,000 D.

20 In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 30,000 D to at or about 40,000 D.

25 In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and at least 30,000D in molecular weight, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is

linear and has a molecular weight that is at or about 30,000 D to at or about 300,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 30,000 D to at or about 100,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 30,000 D to at or about 70,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 30,000 D to at or about 50,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 30,000 D to at or about 40,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at least about 30,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached

to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 30,000 D to at or about 300,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 30,000 D to at or about 100,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 30,000 D to at or about 70,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 30,000 D to at or about 50,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 30,000 D to at or about 40,000 D.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and at least 30,000D in molecular weight, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 30,000 D to at or about 300,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 30,000 D to at or about 100,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 30,000 D to at or about 70,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 30,000 D to at or about 50,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 30,000 D to at or about 40,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at least about 20,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 20,000 D to at or about 300,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 20,000 D to at or about 100,000 D.

5 In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 20,000 D to at or about 70,000 D.

10 In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 20,000 D to at or about 50,000 D.

15 In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 20,000 D to at or about 40,000 D.

20 In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is linear and has a molecular weight that is at or about 20,000 D to at or about 30,000 D.

25 In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and at least 20,000D in molecular weight, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is



linear and has a molecular weight that is at or about 20,000 D to at or about 300,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 20,000 D to at or about 100,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 20,000 D to at or about 70,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 20,000 D to at or about 50,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 20,000 D to at or about 40,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is linear and has a molecular weight that is at or about 20,000 D to at or about 30,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the conjugate contains an antibody fragment selected

from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at least about 20,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 20,000 D to at or about 300,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 20,000 D to at or about 100,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 20,000 D to at or about 70,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 20,000 D to at or about 50,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 20,000 D to at or about 40,000 D.

In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 20,000 D to at or about 30,000 D.

In another preferred embodiment, the invention provides a conjugate containing an

antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and at least 20,000D in molecular weight, and the PEG molecule is attached to the hinge region of the antibody fragment.

5 In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 20,000 D to at or about 300,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

10 In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 20,000 D to at or about 100,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

15 In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 20,000 D to at or about 70,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

20 In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 20,000 D to at or about 50,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

25 In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and has a molecular weight that is at or about 20,000 D to at or about 40,000 D, and the

PEG molecule is attached to the hinge region of the antibody fragment.

In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is  
 5 branched and has a molecular weight that is at or about 20,000 D to at or about 30,000 D, and the PEG molecule is attached to the hinge region of the antibody fragment.

In one aspect, the invention provides any of the above-described conjugates wherein the conjugate contains no more than one antibody fragment. Additionally provided herein is any of the above-described conjugates wherein the conjugate contains one or more antibody fragment(s)  
 10 covalently linked to one or more polymer molecule(s), such as conjugates containing two or more antibody fragments covalently linked together by polymer molecule(s). In one embodiment, a polymer molecule is used to link together two antibody fragments to form a dumbbell-shaped structure. Also encompassed herein are conjugates formed by more than two antibody fragments joined by polymer molecule(s) to form a rosette or other shapes. The  
 15 antibody fragments in such structures can be of the same or different fragment type and can have the same antigen specificity or have different antigen specificities. Such structures can be made by using a polymer molecule derivatized with multiple functional groups permitting the direct attachment, or the attachment by means of bi- or multi-functional linkers, of two or more antibody fragments to the polymer backbone.

In another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising an antigen recognition site that binds to rabbit IL-8 and/or human IL-8. In yet another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising 6G4.2.5LV/L1N35A or  
 20 6G4.2.5LV/L1N35E as defined below. In still another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising 6G4.5.2.5HV11 as defined below. In a further aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising hu6G4.2.5LV/L1N35A or hu6G4.2.5LV/L1N35E as defined below. In an additional aspect, the invention encompasses any

of the above-described conjugates utilizing an antibody fragment comprising hu6G4.2.5HV.

Further encompassed herein are any of the above-described conjugates utilizing an antibody fragment comprising 6G4.2.5LV/L1N35A or 6G4.2.5LV/L1N35E and further comprising the CDRs of 6G4.2.5HV as defined below. Also encompassed herein are any of the above described conjugates utilizing an antibody fragment comprising hu6G4.2.5LV/L1N35A or hu6G4.2.5LV/L1N35E and further comprising hu6G4.2.5HV as defined below. Additionally encompassed herein are any of the above-described conjugates utilizing an antibody fragment comprising 6G4.2.5LV11N35A or 6G4.2.5LV11N35E as defined below. Further provided herein are any of the above-described conjugates utilizing an antibody fragment comprising 6G4.2.5LV11N35A or 6G4.2.5LV11N35E and further comprising 6G4.2.5HV11 as defined below.

In another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising an antigen recognition site that binds to human VEGF.

In another embodiment, the foregoing antibody fragment competes with VEGF receptor for binding to VEGF. Such anti-VEGF antagonistic antibody fragments are used to construct conjugates that are capable of inhibiting one or more of the biological activities of VEGF, for example, its mitogenic or angiogenic activity. Antagonists of VEGF act by interfering with the binding of VEGF to a cellular receptor, by incapacitating or killing cells which have been activated by VEGF, or by interfering with vascular endothelial cell activation after VEGF binding to a cellular receptor. All such points of intervention used by anti-VEGF antagonists are also suitable therapeutic targets for the anti-VEGF antibody fragment-polymer conjugates of the invention. Anti-human VEGF antibodies capable of interfering with the binding of VEGF to a cellular receptor are described in WO 98/45331 published October 15, 1998 (International Application No. PCT/US98/06604 filed April 3, 1998).

In another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising an antigen recognition site that binds to HER2. In another embodiment, the foregoing antibody fragment binds to the extracellular domain of the human ErbB2 (HER2) receptor. In yet another embodiment, the foregoing antibody fragment is

capable of inducing cell death or apoptosis of a HER2-expressing cell. In still another embodiment, the foregoing conjugate utilizing an anti-HER2 antibody fragment further incorporates a radioimaging or radiotherapeutic agent, including radionuclides such as  $^{212}\text{Bi}$ ,  $^{131}\text{I}$ ,  $^{131}\text{In}$ ,  $^{90}\text{Y}$  and  $^{186}\text{Re}$ , or other nonproteinaceous diagnostic label or chemotherapeutic agent, including small molecule toxins such as calicheamicins, maytansinoids, palytoxins, trichothenes, and CC1065.

In another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising an antigen recognition site that binds to human CD20. In another embodiment, the foregoing antibody fragment binds to the extracellular domain of human CD20.

In another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising an antigen recognition site that binds to human CD18. In another embodiment, the foregoing antibody fragment binds to the extracellular domain of human CD18.

In another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising an antigen recognition site that binds to human CD11a. In another embodiment, the foregoing antibody fragment binds to the extracellular domain of human CD11a.

In another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising an antigen recognition site that binds to human IgE. In another embodiment, the foregoing antibody fragment is capable of competing with  $\text{Fc}\epsilon\text{RI}$  receptor for binding to human IgE, i.e. capable of inhibiting the binding of human IgE to the  $\text{Fc}\epsilon\text{RI}$  receptor. In yet another embodiment, the foregoing antibody fragment binds to membrane-bound IgE on the surface of human B-lymphocytes but does not bind to soluble IgE bound to  $\text{Fc}\epsilon\text{RI}$  receptor on the surface of human basophils.

In another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising an antigen recognition site that binds to human Apo-2 receptor. In another embodiment, the foregoing antibody fragment binds to the extracellular

domain of the human Apo-2 receptor. In yet another embodiment, the foregoing antibody fragment is capable of inducing cell death or apoptosis of an Apo-2 receptor-expressing cell.

In another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising an antigen recognition site that binds to human TNF-  
5  $\alpha$ .

In another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising an antigen recognition site that binds to human tissue factor.

In another aspect, the invention encompasses any of the above-described conjugates  
10 utilizing an antibody fragment comprising an antigen recognition site that binds to human  $\alpha_4\beta_7$  integrin. In another embodiment, the foregoing antibody fragment binds to the extracellular region of a human  $\alpha_4\beta_7$  integrin complex.

In another aspect, the invention encompasses any of the above-described conjugates  
15 utilizing an antibody fragment comprising an antigen recognition site that binds to human GPIIb-IIIa integrin. In another embodiment, the foregoing antibody fragment binds to the extracellular region of a human GPIIb-IIIa integrin complex.

In another aspect, the invention encompasses any of the above-described conjugates  
20 utilizing an antibody fragment comprising an antigen recognition site that binds to human CD3. In another embodiment, the foregoing antibody fragment binds to the extracellular domain of human CD3.

In another aspect, the invention encompasses any of the above-described conjugates  
utilizing an antibody fragment comprising an antigen recognition site that binds to human interleukin-2 receptor (IL-2R)  $\alpha$ -chain (T-cell activation antigen or "TAC"). In another  
embodiment, the foregoing antibody fragment binds to the extracellular domain of human TAC.

25 In another aspect, the invention encompasses any of the above-described conjugates  
utilizing an antibody fragment comprising an antigen recognition site that binds to human EGFR. In another embodiment, the foregoing antibody fragment binds to the extracellular domain of human EGFR.

a. Production of Antibody Fragments

Antibody fragments can be produced by any method known in the art. Generally, an antibody fragment is derived from a parental intact antibody.

(i) Antigen Preparation

The antigen to be used for antibody generation can be prepared by any convenient method, such as recombinant methods. Membrane-bound protein antigens can be presented by cell surface expression in recombinant or non-recombinant cells, which cells can be used as immunogens for raising the desired antibody response against the membrane-bound protein antigen. Alternatively, soluble forms of the membrane-bound protein antigen can be generated, such as isolated extracellular domain fragments of membrane-anchored receptor proteins, or variants of such receptor proteins having deleted or inactivated transmembrane domains. In one embodiment, an extracellular domain is fused to the Fc region of an immunoglobulin to form a chimeric protein immunogen.

A protein antigen of interest can be cloned, genetically engineered as desired to add characteristics useful in antibody generation (such as fusion to an immunoglobulin Fc region), and produced in a recombinant expression host cell system according to known methods. In one embodiment, human VEGF-encoding DNA is obtained as described in U.S. Pat. No. 5,332,671 (issued July 26, 1994) and used for production of human VEGF in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of human VEGF from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below. In another embodiment, human VEGF is obtained as described in U.S. Pat. No. 5,332,671.

In one embodiment, soluble HER2-encoding DNA, such as HER2 extracellular domain (ECD)-encoding DNA, is obtained as described in European Patent No. 0 474 727 B1 (granted July 23, 1997) (European regional phase of WO 90/14357 published November 29, 1990) and used for production of HER2 ECD in recombinant host cells according to the same general



methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of human HER2 ECD from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below. In another embodiment, HER2 ECD is obtained as described in EP 0 474 727 B1.

In one embodiment, soluble human CD20-encoding DNA, such as human CD20 extracellular domain (ECD)-encoding DNA, is obtained as described in Tedder et al., "Isolation and Structure of a cDNA Encoding the B1 (CD20) Cell-Surface Antigen of Human B Lymphocytes," Proc. Natl. Acad. Sci. (USA), 85: 208-212 (1988) and used for production of CD20 ECD in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of human CD20 ECD from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below.

In one embodiment, soluble human CD11a-encoding DNA, such as human CD11a I-domain-encoding DNA, is obtained as described in van Kooyk et al., J. Exp. Med., 183(3): 1247-1252 (1996), Edwards et al., J. Biol. Chem., 270(21): 12635-12640 (1995), or Champe et al., J. Biol. Chem., 270: 1388-1394 (1995), and used for production of CD11a I-domain in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of human CD11a I-domain from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below.

In one embodiment, soluble human CD18-encoding DNA, such as human CD18 extracellular domain (ECD)-encoding DNA, is obtained as described in Kishimoto et al., "Cloning of the beta subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family," Cell, 48:681-690 (1987) and used for production of CD18 ECD in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by

recovery and purification of human CD18 ECD from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below.

In one embodiment, human membrane-bound IgE extracellular domain-encoding DNA is obtained as described in U.S. Pat. No. 5,091,131 (issued February 25, 1992) and used for production of human membrane-bound IgE ECD in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of human membrane-bound IgE ECD from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below. In another embodiment, human membrane-bound IgE ECD is obtained as described in U.S. Pat. No. 5,091,131.

In one embodiment, soluble human Apo-2 receptor-encoding DNA, such as human Apo-2 receptor extracellular domain (ECD)-encoding DNA, is obtained as described in WO 98/51793 (published November 19, 1998) (International Application No. PCT/US98/09704 filed May 14, 1998) and used for production of human Apo-2 receptor ECD in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of human Apo-2 receptor ECD from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below.

In one embodiment, human TNF- $\alpha$ -encoding DNA is obtained as described in Pennica et al., *Nature*, 512: 721 (1984) or U.S. Pat. No. 4,650,674 (issued March 17, 1987) and used for production of human TNF- $\alpha$  in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of human TNF- $\alpha$  from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below. In another embodiment, human TNF- $\alpha$  is obtained as described in U.S. Pat. No. 4,650,674.

In one embodiment, human tissue factor-encoding DNA is obtained as described in

European Patent No. 0 278 776 B1 (granted May 28, 1997) and used for production of human tissue factor in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of human tissue factor from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below. In another embodiment, human tissue factor is obtained as described in European Patent No. 0 278 776 B1.

In one embodiment, soluble human  $\alpha_4$  integrin-encoding DNA and soluble human  $\beta_7$  integrin encoding DNA, such as human  $\alpha_4$  integrin extracellular domain (ECD)-encoding DNA along with human  $\beta_7$  integrin extracellular domain (ECD)-encoding DNA, are obtained as described in Takada et al., "The primary structure of the  $\alpha_4$  subunit of VLA-4: Homology to other integrins and a possible cell-cell adhesion function", EMBO J., 8: 1361-1368 (1989) and Yuan et al., "Cloning and sequence analysis of a novel  $\beta_2$ -related integrin transcript from T lymphocytes: homology of integrin cysteine-rich repeats to domain III of laminin B chains", International Immunology, 2: 1097-1108 (1990), respectively, and used for co-production of human  $\alpha_4$  integrin ECD and human  $\beta_7$  integrin ECD in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of  $\alpha_4$  ECD- $\beta_7$  ECD complex from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below.

In one embodiment, soluble human GPIIb-encoding DNA and soluble human GPIIIa-encoding DNA, such as human GPIIb extracellular domain (ECD)-encoding DNA along with human GPIIIa extracellular domain (ECD)-encoding DNA, are obtained as described in U.S. Pat. No. 5,726,037 (issued March 10, 1998) and used for co-production of human GPIIb ECD and human GPIIIa ECD in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of GPIIb ECD-GPIIIa ECD complex from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments

in Section (II)(4)(F) below. Alternatively, human GPIIb-IIIa complex can be produced and secreted from recombinant host cells as described in Example 3 of U.S. Pat. No. 5,726,037.

In one embodiment, soluble human epidermal growth factor receptor (EGFR)-encoding DNA, such as human EGFR extracellular domain (ECD)-encoding DNA, is obtained as described in Ullrich et al., Nature, 309: 418-425 (1984) and used for production of human EGFR ECD in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of human EGFR ECD from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below.

In one embodiment, soluble human CD3-encoding DNA, such as human CD3 extracellular domain (ECD)-encoding DNA, is obtained as described in van den Elsen et al., "Isolation of cDNA clones encoding the 20K T3 glycoprotein of human T-cell receptor complex," Nature, 312:413-418 (1984) and used for production of human CD3 ECD in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of human CD3 ECD from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below.

In one embodiment, soluble human interleukin-2 receptor (IL-2R)  $\alpha$ -chain (T-cell activation antigen or "TAC")-encoding DNA, such as human TAC extracellular domain (ECD)-encoding DNA, is obtained as described in Leonard et al., Science, 230: 633-639 (1985) and used for production of human TAC ECD in recombinant host cells according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4) below, followed by recovery and purification of human TAC ECD from recombinant host cell culture according to the same general methods that are described for antibodies and antibody fragments in Section (II)(4)(F) below.

#### (ii) Polyclonal Antibodies

The parental antibody can be generated by raising polyclonal sera against the desired

antigen by multiple subcutaneous (sc) or intraperitoneal (ip) injections of antigen and an adjuvant, such as monophosphoryl lipid A (MPL)/trehalose dicrynomycolate (TDM) (Ribi Immunochem. Research, Inc., Hamilton, MT), at multiple sites. Two weeks later the animals are boosted. 7 to 14 days later animals are bled and the serum is assayed for anti-antigen titer.

- 5 Animals are boosted until titer plateaus. Sera are harvested from animals, and polyclonal antibodies are isolated from sera by conventional immunoglobulin purification procedures, such as protein A-Sepharose chromatography, hydroxylapatite chromatography, gel filtration, dialysis, or antigen affinity chromatography. The desired antibody fragments can be generated from purified polyclonal antibody preparations by conventional enzymatic methods, e.g. F(ab')<sub>2</sub>
- 10 fragments are produced by pepsin cleavage of intact antibody, and Fab fragments are produced by briefly digesting intact antibody with papain.

### (iii) Monoclonal Antibodies

Alternatively, antibody fragments are derived from monoclonal antibodies generated against the desired antigen. Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium),

which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOP-21 and M.C.-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

In one embodiment, anti-human VEGF monoclonal antibody is obtained as described in WO 98/45331 (published October 15, 1998) (International Application No. PCT/US98/06604 filed April 3, 1998).

In another embodiment, anti-HER2 monoclonal antibody is obtained as described in U.S. Patent No. 5,725,856 (issued March 10, 1998) or International Application No. CT/US98/26266 (filed December 10, 1998).

In another embodiment, anti-human CD20 monoclonal antibody is obtained as described in WO 94/11026 (published May 26, 1994) (International Application No. PCT/US93/10953 filed November 12, 1993).

In another embodiment, anti-human CD18 monoclonal antibody is obtained as described in U.S. Pat. No. 5,622,700 (issued April 22, 1997). In yet another embodiment, anti-human CD18 monoclonal antibody is obtained as described in WO 97/26912 (published July 31, 1997) (International Application No. PCT/US97/00492 filed January 11, 1997).

In another embodiment, anti-human CD11a monoclonal antibody is obtained as described in U.S. Pat. No. 5,622,700. In yet another embodiment, anti-human CD11a monoclonal antibody is obtained as described in WO 98/23761 (published June 4, 1998) (International Application No. PCT/US97/19041 filed October 20, 1997).

In another embodiment, anti-human IgE monoclonal antibody is obtained as described in U.S. Pat. No. 5,714,338 (issued February 3, 1998). In yet another embodiment, anti-human IgE monoclonal antibody is obtained as described in U.S. Pat. No. 5,091,313 (issued February 25, 1992). In still another embodiment, anti-human IgE monoclonal antibody is obtained as described in WO 93/04173 (published March 4, 1993) (International Application No. PCT/US92/06860 filed August 14, 1992). In an additional embodiment, anti-human IgE monoclonal antibody is obtained as described in International Application No. PCT/US98/13410 (filed June 30, 1998). In a further aspect, the invention contemplates the use of anti-human IgE monoclonal antibody capable of competing with Fc $\epsilon$ RI receptor for binding to human IgE, i.e. capable of inhibiting the binding of human IgE to the Fc $\epsilon$ RI receptor. Such anti-IgE monoclonal antibodies can be selected and identified by any convenient screening method, such an assay for

inhibition of IgE-induced basophil cell sensitization as described in U.S. Pat. No. 5,714,338.

In another embodiment, anti-human Apo-2 receptor monoclonal antibody is obtained as described in WO 98/51793 (published November 19, 1998) (International Application No. PCT/US98/09704 filed May 14, 1998). In a further embodiment, the invention contemplates the use of anti-human Apo-2 receptor monoclonal antibody capable of activating the human Apo-2 receptor. Such anti-Apo-2 monoclonal antibodies can be selected and identified by any convenient screening method, such as an assay for induction of Apo-2 mediated 9D cell apoptosis as described in Example 10 of WO 98/51793.

In another embodiment, anti-human TNF- $\alpha$  monoclonal antibody is obtained as described in U.S. Pat. No. 5,672,347 (issued September 30, 1997).

In another embodiment, anti-human tissue factor monoclonal antibody is obtained as described in European Patent No. 0 420 937 B1 (granted November 9, 1994).

In another embodiment, anti-human  $\alpha_4\beta_7$  integrin monoclonal antibody is obtained as described in WO 98/06248 (published February 19, 1998) (International Application No. PCT/US97/13884 filed August 6, 1997).

In another embodiment, anti-human EGFR monoclonal antibody is obtained as described in WO 96/40210 (published December 19, 1996) (International Application No. PCT/US96/9847 filed June 7, 1996).

In another embodiment, anti-human CD3 monoclonal antibody is obtained as described in U.S. Pat. No. 4,515,893 (issued May 7, 1985).

In another embodiment, anti-human TAC monoclonal antibody is obtained as described in U.S. Pat. No. 5,693,762 (issued December 2, 1997).

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise



produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of antibody-encoding DNA include Skerra et al., Curr. Opinion in Immunol., 5: 256 (1993) and Pluckthun, Immunol. Revs., 130: 151 (1992).

5 In a preferred embodiment, the antibody fragment is derived from a humanized antibody. Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. It will be appreciated that variable  
10 domain sequences obtained from any non-human animal phage display library-derived Fv clone or from any non-human animal hybridoma-derived antibody clone provided as described herein can serve as the "import" variable domain used in the construction of the humanized antibodies of the invention. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, *Nature*, **321**: 522 (1986); Riechmann *et al.*, *Nature*, **332**: 323  
15 (1988); Verhoeven *et al.*, *Science*, **239**: 1534 (1988)), by substituting non-human animal, e.g. rodent, CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly *et al.*, *supra*), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are  
20 typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in non-human animal, e.g. rodent, antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a non-human animal, e.g. rodent, antibody is  
25 screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the non-human animal is then accepted as the human framework (FR) for the humanized antibody (Sims *et al.*, *J. Immunol.*, **151**: 2296 (1993); Chothia and Lesk, *J. Mol. Biol.*, **196**: 901 (1987)). Another method uses a particular framework derived

from the consensus sequence of all human antibodies of a particular subgroup light or heavy chains. The same framework can be used for several different humanized antibodies (Carter *et al.*, *Proc. Natl. Acad. Sci USA*, **89**: 4285 (1992); Presta *et al.*, *J. Immunol.*, **151**: 2623 (1993)). It is also important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind to its antigen. In this way, FR residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

In addition, antibody fragments for use herein can be derived from human monoclonal antibodies. Human monoclonal antibodies against the antigen of interest can be made by the hybridoma method. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described, for example, by Kozbor *J. Immunol.*, **133**: 3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner *et al.*, *J. Immunol.*, **147**: 86 (1991).

It is now possible to produce transgenic animals (e.g. mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line

immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci USA*, **90**: 2551 (1993); Jakobovits *et al.*, *Nature*, **362**: 255 (1993); Bruggermann *et al.*, *Year in Immunol.*, **7**: 33 (1993).

Alternatively, phage display technology (McCafferty *et al.*, *Nature* 348:552 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson *et al.*, *Current Opinion in Structural Biology* 3:564 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al.*, *Nature* 352:624 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks *et al.*, *J. Mol. Biol.* 222:581 (1991), or Griffith *et al.*, *EMBO J.* 12:725 (1993). In a natural immune response, antibody genes accumulate mutations at a high rate (somatic hypermutation). Some of the changes introduced will confer higher affinity, and B cells displaying high-affinity surface immunoglobulin are preferentially replicated and differentiated during subsequent antigen challenge. This natural process can be mimicked by employing the technique known as "chain shuffling" (Marks *et al.*, *Bio/Technol.* 10:779 (1992)). In this method, the affinity of "primary" human antibodies obtained by phage display can be improved by sequentially replacing the heavy and light chain V region genes with repertoires of naturally occurring variants (repertoires) of V domain genes obtained from unimmunized donors. This

technique allows the production of antibodies and antibody fragments with affinities in the nM range. A strategy for making very large phage antibody repertoires has been described by Waterhouse et al., Nucl. Acids Res. 21:2265 (1993).

Gene shuffling can also be used to derive human antibodies from non-human, e.g. rodent, antibodies, where the human antibody has similar affinities and specificities to the starting non-human antibody. According to this method, which is also called "epitope imprinting", either the heavy or light chain variable region of a non-human antibody fragment obtained by phage display techniques as described above is replaced with a repertoire of human V domain genes, creating a population of non-human chain/human chain scFv or Fab chimeras. Selection with antigen results in isolation of a non-human chain/human chain chimeric scFv or Fab wherein the human chain restores the antigen binding site destroyed upon removal of the corresponding non-human chain in the primary phage display clone, i.e. the epitope governs (imprints) the choice of the human chain partner. When the process is repeated in order to replace the remaining non-human chain, a human antibody is obtained (see PCT WO 93/06213 published April 1, 1993). Unlike traditional humanization of non-human antibodies by CDR grafting, this technique provides completely human antibodies, which have no FR or CDR residues of non-human origin.

The invention also encompasses the use of bispecific and heteroconjugate antibody fragments having specificities for at least two different antigens. Bispecific and heteroconjugate antibodies can be prepared as full length antibodies or as antibody fragments (e.g. F(ab')<sub>2</sub> bispecific antibody fragments). Antibody fragments having more than two valencies (e.g. trivalent or higher valency antibody fragments) are also contemplated for use herein. Bispecific antibodies, heteroconjugate antibodies, and multi-valent antibodies can be prepared as described in Section (II)(3)(C) below.

As described above, DNA encoding the monoclonal antibody or antibody fragment of interest can be isolated from its hybridoma or phage display clone of origin, and then manipulated to create humanized and/or affinity matured constructs. In addition, known techniques can be employed to introduce an amino acid residue or residues into any desired location on the polypeptide backbone of the antibody fragment, e.g. a cysteine residue placed in

the hinge region of the heavy chain, thereby providing a site for specific attachment of polymer molecule(s). In one embodiment, the native cysteine residue in either the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains is substituted with another amino acid, such as serine, in order to leave the partner  
 5 cysteine residue in the opposite chain with a free suflhydryl for specific attachment of polymer molecule.

Upon construction of the desired antibody or antibody fragment-encoding clone, the clone can be used for recombinant production of the antibody fragment as described in Section (II)(4) below. Finally, the antibody or antibody fragment product can be recovered from host cell  
 10 culture and purified as described in Section (II)(4)(F) below. In the case of embodiments utilizing an antibody fragment engineered to lack a cysteine residue that ordinarily forms the disulfide bridge between the light and heavy chains as described above, preferred recombinant production systems include bacterial expression and product recovery procedures utilizing the low pH osmotic shock method described in the "Alternative Fab'-SH Purification" section of  
 15 Example T below. If a full length antibody is produced, the desired antibody fragment can be obtained therefrom by subjecting the intact antibody to enzymatic digestion according to known methods, e.g. as described in Section (II)(4)(G) below.

#### b. Construction of Antibody Fragment-Polymer Conjugates

The antibody fragment-polymer conjugates of the invention can be made by derivatizing the desired antibody fragment with an inert polymer. It will be appreciated that any inert polymer which provides the conjugate with the desired apparent size or which has the selected actual MW as taught herein is suitable for use in constructing the antibody fragment-polymer  
 20 conjugates of the invention.

Many inert polymers are suitable for use in pharmaceuticals. See, e.g., Davis et al., Biomedical Polymers: Polymeric Materials and Pharmaceuticals for Biomedical Use, pp.441-451 (1980). In all embodiments of the invention, a non-proteinaceous polymer is used. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e., a polymer not

otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or *in vitro* methods are also useful, as are polymers which are isolated from native sources. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyalkylene ethers such as polyethylene glycol (PEG); polyoxyalkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronics); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g. polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextrane sulfate, dextran, dextrans, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, e.g. hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol; heparin or heparon. The polymer prior to cross-linking need not be, but preferably is, water soluble, but the final conjugate must be water soluble. Preferably, the conjugate exhibits a water solubility of at least about 0.01 mg/ml, and more preferably at least about 0.1 mg/ml, and still more preferably at least about 1 mg/ml. In addition, the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if the conjugate is intended to be administered by such routes.

In one embodiment, the polymer contains only a single group which is reactive. This helps to avoid cross-linking of protein molecules. However, it is within the scope herein to maximize reaction conditions to reduce cross-linking, or to purify the reaction products through gel filtration or ion exchange chromatography to recover substantially homogenous derivatives.

In other embodiments, the polymer contains two or more reactive groups for the purpose of linking multiple antibody fragments to the polymer backbone. Again, gel filtration or ion exchange chromatography can be used to recover the desired derivative in substantially homogeneous form.

The molecular weight of the polymer can range up to about 500,000 D, and preferably is at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. The molecular weight chosen can depend upon the effective size of the conjugate to be achieved, the nature (e.g. structure, such as linear or branched) of the polymer, and the degree of derivatization, i.e. the number of polymer molecules per antibody fragment, and the polymer attachment site or sites on the antibody fragment.

The polymer can be covalently linked to the antibody fragment through a multifunctional crosslinking agent which reacts with the polymer and one or more amino acid residues of the antibody fragment to be linked. However, it is also within the scope of the invention to directly crosslink the polymer by reacting a derivatized polymer with the antibody fragment, or vice versa.

The covalent crosslinking site on the antibody fragment includes the N-terminal amino group and epsilon amino groups found on lysine residues, as well as other amino, imino, carboxyl, sulfhydryl, hydroxyl or other hydrophilic groups. The polymer may be covalently bonded directly to the antibody fragment without the use of a multifunctional (ordinarily bifunctional) crosslinking agent. Covalent binding to amino groups is accomplished by known chemistries based upon cyanuric chloride, carbonyl diimidazole, aldehyde reactive groups (PEG alkoxide plus diethyl acetal of bromoacetaldehyde; PEG plus DMSO and acetic anhydride, or PEG chloride plus the phenoxide of 4-hydroxybenzaldehyde, activated succinimidyl esters, activated dithiocarbonate PEG, 2,4,5-trichlorophenylchloroformate or P-nitrophenylchloroformate activated PEG.) Carboxyl groups are derivatized by coupling PEG-amine using carbodiimide. Sulfhydryl groups are derivatized by coupling to maleimido-substituted PEG (e.g. alkoxy-PEG amine plus sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) as described in WO 97/10847 published March 27, 1997, or PEG-maleimide commercially available from Shearwater Polymers, Inc., Huntsville, AL). Alternatively, free amino groups on the antibody fragment (e.g. epsilon amino groups on lysine residues) can be thiolated with 2-imino-thiolane (Traut's reagent) and then coupled to maleimide-containing derivatives of PEG as described in Pedley et al., Br. J. Cancer, 70: 1126-1130 (1994).

The polymer will bear a group which is directly reactive with an amino acid side chain, or the N- or C-terminus of the polypeptide linked, or which is reactive with the multifunctional cross-linking agent. In general, polymers bearing such reactive groups are known for the preparation of immobilized proteins. In order to use such chemistries here, one should employ a water soluble polymer otherwise derivatized in the same fashion as insoluble polymers heretofore employed for protein immobilization. Cyanogen bromide activation is a particularly useful procedure to employ in crosslinking polysaccharides.

"Water soluble" in reference to the starting polymer means that the polymer or its reactive intermediate used for conjugation is sufficiently water soluble to participate in a derivatization reaction.

The degree of substitution with such a polymer will vary depending upon the number of reactive sites on the antibody fragment, the molecular weight, hydrophilicity and other characteristics of the polymer, and the particular antibody fragment derivatization sites chosen. In general, the conjugate contains from 1 to about 10 polymer molecules, but greater numbers of polymer molecules attached to the antibody fragments of the invention are also contemplated. The desired amount of derivatization is easily achieved by using an experimental matrix in which the time, temperature and other reaction conditions are varied to change the degree of substitution, after which the level of polymer substitution of the conjugates is determined by size exclusion chromatography or other means known in the art.

The polymer, e.g. PEG, is cross-linked to the antibody fragment by a wide variety of methods known *per se* for the covalent modification of proteins with nonproteinaceous polymers such as PEG. Certain of these methods, however, are not preferred for the purposes herein. Cyanuronic chloride chemistry leads to many side reactions, including protein cross-linking. In addition, it may be particularly likely to lead to inactivation of proteins containing sulfhydryl groups. Carbonyl diimidazole chemistry (Beauchamp *et al.*, Anal Biochem. 131, 25-33 [1983]) requires high pH (>8.5), which can inactivate proteins. Moreover, since the "activated PEG" intermediate can react with water, a very large molar excess of "activated PEG" over protein is required. The high concentrations of PEG required for the carbonyl diimidazole chemistry also



led to problems in purification, as both gel filtration chromatography and hydrophilic interaction chromatography are adversely affected. In addition, the high concentrations of "activated PEG" may precipitate protein, a problem that *per se* has been noted previously (Davis, U.S. Patent No. 4,179,337). On the other hand, aldehyde chemistry (Royer, U.S. Patent No. 4,002,531) is more efficient since it requires only a 40-fold molar excess of PEG and a 1-2 hr incubation. However, the manganese dioxide suggested by Royer for preparation of the PEG aldehyde is problematic "because of the pronounced tendency of PEG to form complexes with metal-based oxidizing agents" (Harris *et al.*, *J. Polym. Sci. Polym. Chem. Ed.* 22, 341-52 [1984]). The use of a Moffatt oxidation, utilizing DMSO and acetic anhydride, obviates this problem. In addition, the sodium borohydride suggested by Royer must be used at high pH and has a significant tendency to reduce disulfide bonds. In contrast, sodium cyanoborohydride, which is effective at neutral pH and has very little tendency to reduce disulfide bonds is preferred. In another preferred embodiment, maleimido-activated PEG is used for coupling to free thiols on the antibody fragment.

Functionalized PEG polymers to modify the antibody fragments of the invention are available from Shearwater Polymers, Inc. (Huntsville, AL). Such commercially available PEG derivatives include, but are not limited to, amino-PEG, PEG amino acid esters, PEG-hydrazide, PEG-thiol, PEG-succinate, carboxymethylated PEG, PEG-propionic acid, PEG amino acids, PEG succinimidyl succinate, PEG succinimidyl propionate, succinimidyl ester of carboxymethylated PEG, succinimidyl carbonate of PEG, succinimidyl esters of amino acid PEGs, PEG-oxycarbonylimidazole, PEG-nitrophenyl carbonate, PEG tresylate, PEG-glycidyl ether, PEG-aldehyde, PEG vinylsulfone, PEG-maleimide, PEG-orthopyridyl-disulfide, heterofunctional PEGs, PEG vinyl derivatives, PEG silanes, and PEG phospholides. The reaction conditions for coupling these PEG derivatives will vary depending on the protein, the desired degree of PEGylation, and the PEG derivative utilized. Some factors involved in the choice of PEG derivatives include: the desired point of attachment (such as lysine or cysteine R-groups), hydrolytic stability and reactivity of the derivatives, stability, toxicity and antigenicity of the linkage, suitability for analysis, etc. Specific instructions for the use of any particular

derivative are available from the manufacturer.

The conjugates of this invention are separated from the unreacted starting materials by gel filtration or ion exchange HPLC. Heterologous species of the conjugates are purified from one another in the same fashion.

5 The conjugates may also be purified by ion-exchange chromatography. The chemistry of many of the electrophilically activated PEG's results in a reduction of amino group charge of the PEGylated product. Thus, high resolution ion exchange chromatography can be used to separate the free and conjugated proteins, and to resolve species with different levels of PEGylation. In fact, the resolution of different species (e.g. containing one or two PEG residues) is also possible  
10 due to the difference in the ionic properties of the unreacted amino acids. In one embodiment, species with difference levels of PEGylation are resolved according to the methods described in WO 96/34015 (International Application No. PCT/US96/05550 published October 31, 1996).

In a preferred embodiment, the conjugate is generated by utilizing the derivatization and purification methods described in Section (T) of the Examples below.

15 In one aspect, the invention provides any of the above-described conjugates formed by its component parts, i.e. one or more antibody fragment(s) covalently attached to one or more polymer molecule(s), without any extraneous matter in the covalent molecular structure of the conjugate.

20 c. Other Derivatives of Large Effective Size Conjugates

In another aspect, any of the above-described conjugates can be modified to contain one or more component(s) in addition to the antibody fragment component(s) and polymer component(s) that form the conjugate, wherein the modification does not alter the essential functional property of the conjugate, namely, the substantially improved serum half-life, MRT  
25 and/or serum clearance rate as compared to that of the parental antibody fragment from which the conjugate is derived. In one embodiment, the invention provides any of the above-described conjugates modified to incorporate one or more nonproteinaceous functional group(s). For example, the conjugate can be modified to incorporate nonproteinaceous labels or reporter

molecules, such as radiolabels, including any radioactive substance used in medical treatment or imaging or used as an effector function or tracer in an animal model, such as radioisotopic labels  $^{99}\text{Tc}$ ,  $^{90}\text{Y}$ ,  $^{111}\text{In}$ ,  $^{32}\text{P}$ ,  $^{14}\text{C}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$ ,  $^{131}\text{I}$ ,  $^{11}\text{C}$ ,  $^{15}\text{O}$ ,  $^{13}\text{N}$ ,  $^{18}\text{F}$ ,  $^{35}\text{S}$ ,  $^{51}\text{Cr}$ ,  $^{57}\text{To}$ ,  $^{226}\text{Ra}$ ,  $^{60}\text{Co}$ ,  $^{59}\text{Fe}$ ,  $^{75}\text{Se}$ ,  $^{152}\text{Eu}$ ,  $^{67}\text{Cu}$ ,  $^{217}\text{Ci}$ ,  $^{211}\text{At}$ ,  $^{212}\text{Pb}$ ,  $^{47}\text{Sc}$ ,  $^{109}\text{Pd}$ ,  $^{234}\text{Th}$ ,  $^{40}\text{K}$ , and the like, non-radioisotopic labels such as  $^{157}\text{Gd}$ ,  $^{55}\text{Mn}$ ,  $^{52}\text{Tr}$ ,  $^{56}\text{Fe}$ , etc., fluorescent or chemiluminescent labels, including fluorophores such as rare earth chelates, fluorescein and its derivatives, rhodamine and its derivatives, isothiocyanate, phycoerythrin, phycocyanin, allophycocyanin, o-phthalaldehyde, fluorescamine,  $^{152}\text{Eu}$ , dansyl, umbelliferone, luciferin, luminal label, isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, an aequorin label, 2,3-dihydrophthalazinediones, biotin/avidin, spin labels, stable free radicals, and the like.

Conventional methods are available to bind these labels covalently to the polypeptide antibody fragment or polymer component of the conjugate. In one aspect, any conjugate of the invention is modified by derivatizing the antibody fragment component with any of the above-described non-proteinaceous labels, wherein the label is directly or indirectly (through a coupling agent) attached to the antibody fragment, and wherein such derivatization of the antibody fragment does not contribute or introduce any polymer moiety into the molecular structure of the conjugate. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like can be used to tag the antibody fragment with the above-described fluorescent or chemiluminescent labels. See, for example, U.S. Pat. No. 3,940,475 (fluorimetry), Morrison, *Meth. Enzymol.*, **32b**, 103 (1974), Svyanen et al., *J. Biol. Chem.*, **284**, 3762 (1973), and Bolton and Hunter, *Biochem. J.*, **133**, 529 (1973).

In the case of embodiments utilizing radiolabels, both direct and indirect labeling can be used to incorporate the selected radionuclide into the conjugate. As used herein in the context of radiolabeling, the phrases "indirect labeling" and "indirect labeling approach" both mean that a chelating agent is covalently attached to the antibody fragment moiety or polymer moiety of the conjugate and at least one radionuclide is inserted into the chelating agent. Preferred chelating agents and radionuclides are set forth in Srivastava, S.C. and Mease, R.C., "Progress in Research on Ligands, Nuclides and Techniques for Labeling Monoclonal Antibodies," *Nucl. Med. Bio.*,

18(6): 589-603 (1991). A particularly preferred chelating agent is 1-isothiocycmatobenzyl-3-methyldiothelene triaminepent acetic acid ("MX-DTPA"). As used herein in the context of radiolabeling, the phrases "direct labeling" and "direct labeling approach" both mean that a radionuclide is covalently attached directly to the antibody fragment moiety (typically via an amino acid residue) or to the polymer moiety of the conjugate. Preferred radionuclides for use in direct labeling of conjugate are provided in Srivagtava and Mease, *supra*. In one embodiment, the conjugate is directly labeled with <sup>131</sup>I covalently attached to tyrosine residues. In another embodiment, the antibody fragment component of the conjugate is directly or indirectly labeled with any of the above-described radiolabels, wherein such labeling of the antibody fragment does not contribute or introduce any polymer moiety into the molecular structure of the conjugate.

In another embodiment, the conjugate can be modified to incorporate one or more small molecule toxins, such as a calicheamicin, a maytansine (US Patent 5,208,020, expressly incorporated herein by reference), palytoxin, a trichothene, and CC1065. For example, the conjugate of the invention can be derivatized with one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antibody molecule). Maytansine can be converted to May-ss-Me, which can be reduced to May-SH3 and reacted with modified antibody fragment to generate a maytansinoid-derivatized antibody fragment moiety in the conjugate.

In yet another embodiment, the antibody fragment in the conjugate is derivatized with one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, <sup>1</sup><sub>1</sub>, <sup>1</sup><sub>2</sub>, <sup>1</sup><sub>3</sub>, N-acetyl-<sup>1</sup><sub>1</sub>, PSAG and <sup>1</sup><sub>1</sub> (Hinman *et al.*, *Cancer R.* 53:3336-3342 [1993]; and Lode *et al.*, *Cancer R.* 58:2925-2928 [1998]).

#### d. Therapeutic Compositions and Administration of Large Effective Size Conjugates

The conjugate of the invention is useful for treating the disease indications that are treated with the parent intact antibody. In one aspect, the invention provides the use of conjugates derived from a parental antibody that binds to an effector molecule selected from the group

consisint of human VEGF, HER2, human CD20, human CD18, human CD11a, human IgE, human Apo-2 receptor, human TNF- $\alpha$ , human tissue factor, human  $\alpha_4\beta_7$  integrin, human GPIIb-IIIa integrin, human EGFR, human CD3, human IL-2R  $\alpha$ -chain, and human IL-8 in the treatment of a disease that is mediated by the effector molecule.

5 (i) VEGF-Mediated Disorders

In one embodiment, the invention provides a method for treating a VEGF-mediated disease in a human patient with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human VEGF. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of VEGF-mediated disorders, including pathologies supported by blood vessel proliferation, i.e. angiogenesis, in a manner similar to the application of anti-VEGF antibodies in the treatment of such disease indications that is known in the art, which treatment indications include solid tumors ((Kim *et al. Nature* 362:841-844 (1993); Warren *et al. J. Clin. Invest.* 95:1789-1797 (1995); Borgström *et al. Cancer Res.* 56:4032-4039 (1996); and Melnyk *et al. Cancer Res.* 56:921-924 (1996)) and intraocular neovascular syndromes such as proliferative retinopathies and age-related macular degeneration (AMD) (Adamis *et al. Arch. Ophthalmol.* 114:66-71 (1996)).

As shown in the Examples below, the conjugates of the invention approximate the in vivo pharmacokinetics (e.g. serum half-life, clearance and mean residence time as shown in Figs. 72-73 and in Example AB below) and the in vivo therapeutic efficacy (e.g. the treatment of solid tumors as shown in Fig. 74 and in Example AC below) of full length anti-VEGF monoclonal antibody. Since conjugates of the invention derived from anti-VEGF antibodies and fragments display the same or substantially similar in vivo activities as full length anti-VEGF monoclonal antibody across a range of different parameters, including pharmacokinetic characteristics and therapeutic endpoints in an animal tumor model, the data support the efficacy of the conjugates in the same broad spectrum of neovascular disease indications that responds to full length anti-VEGF antibody treatment.

As noted above, any conjugate described in this Section (II) that is derived from an anti-VEGF antibody or fragment can be advantageously utilized in a method of treating a VEGF-

mediated disease or disorder, such as neovascular disorders. In one embodiment, the invention provides a method of treating a neovascular disorder in a human patient comprising administering to the patient an effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that  
5 binds to human VEGF.

In another embodiment, the invention provides a method of treating a solid tumor disorder in a human patient comprising administering to the patient an effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human VEGF. In yet another embodiment, the  
10 solid tumor disorder in the foregoing method is selected from the group consisting of breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma,  
15 Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.  
20

In still another embodiment, the invention provides a method of treating an intraocular neovascular disorder in a human patient comprising administering to the patient an effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human VEGF. In a further  
25 embodiment, the intraocular neovascular disorder is selected from the group consisting of diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, and age-related macular degeneration.

In another embodiment, the invention provides a method of inhibiting angiogenesis in a

human patient comprising administering to the patient an effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human VEGF.

(ii) Disorders Mediated by HER2-Expressing Cells

In one embodiment, the invention provides a method for treating a human disease mediated by HER2-expressing cells with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to HER2. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of HER2-expressing cell-mediated disorders, including pathologies supported by the proliferation of cells expressing HER2, such as cancers characterized by overexpression of HER2, in a manner similar to the application of full length anti-HER2 antibodies in the treatment of such disease indications that is known in the art, which treatment indications include HER2-overexpressing breast, ovarian and lung cancers.

In one embodiment, the invention provides a method of treating a HER2-expressing cell mediated disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to HER2. The disorder can be a HER2-expressing cell proliferative disorder, including a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, *e.g.* a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. In addition, the invention contemplates the use of the foregoing conjugate in place of full length anti-HER2 antibody in the treatment of HER2-overexpressing cancers as described in U.S. Pat. No. 5,725,856 or International Patent Application No. PCT/US98/26266.

(iii) Disorders Mediated by CD20-Expressing Cells

In one embodiment, the invention provides a method for treating a human disease mediated by CD20-expressing cells with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human CD20. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of CD20-expressing cell-mediated disorders, including pathologies supported by the proliferation of CD20-expressing cells, such as cancers of CD20-expressing cells, in a manner similar to the application of full length anti-CD20 antibodies in the treatment of such disease indications that is known in the art, which treatment indications include B-lymphocytic lymphomas.

In one embodiment, the invention provides a method of treating a disorder in a human patient mediated by a CD20-expressing cell, comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human CD20. In another embodiment, the CD20-expressing cell-mediated disorder is a B-lymphocyte proliferative disorder, such as B-lymphocytic lymphoma. In addition, the invention contemplates the use of the foregoing conjugate in place of full length anti-CD20 antibody in the treatment of B-lymphocyte proliferative disorders as described in WO 94/11026 (published May 26, 1994) (International Application No. PCT/US93/10953 filed November 12, 1993).

(iv) Disorders Mediated by CD18-Expressing Cells

In one embodiment, the invention provides a method for treating a human disease mediated by CD18-expressing cells with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human CD18. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of CD18-expressing cell-mediated disorders, including pathologies supported by leukocyte adhesion, in a manner similar to the application of full length anti-CD18 antibodies in the treatment of such disease indications that is known in the art, which treatment indications include acute myocardial infarction and stroke.

In one embodiment, the invention provides a method of treating a disorder in a human patient mediated by a CD18-expressing cell, comprising administering to the patient a



therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human CD18. In another embodiment, the CD18-expressing cell-mediated disorder is an inflammatory disorder, such as an ischemic reperfusion disorder, including acute myocardial infarction and stroke. In addition, the invention contemplates the use of the foregoing conjugate in place of full length anti-CD18 antibody in the treatment of stroke as described in WO 97/26912.

In another embodiment, the invention provides a method of treating a LFA-1-mediated disorder in a human, comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human CD18. In addition, the invention contemplates the use of the foregoing conjugate in place of full length anti-CD18 antibody in the treatment of an LFA-1-mediated disorder, such as psoriasis and graft rejection, in a human patient as described in U.S. Pat. No. 5,622,700.

(v) Disorders Mediated by CD11a-Expressing Cells

In one embodiment, the invention provides a method for treating a human disease mediated by a CD11a-expressing cell with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human CD11a. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of CD11a-expressing cell-mediated disorders, including pathologies supported by leukocyte adhesion, in a manner similar to the application of full length anti-CD11a antibodies in the treatment of such disease indications that is known in the art, which treatment indications include psoriasis, asthma, graft rejection, and multiple sclerosis.

In one embodiment, the invention provides a method of treating a disorder in a human patient mediated by a CD11a-expressing cell, comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human CD11a.

In another embodiment, the invention provides a method of treating an inflammatory

disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human CD11a. In another embodiment, the inflammatory disorder is psoriasis.

5 In another embodiment, the invention provides a method of treating an immune disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human CD11a. In still another embodiment, the immune disorder is graft rejection. In a further embodiment, the immune  
10 disorder is multiple sclerosis.

In another embodiment, the invention provides a method of treating asthma in a human patient comprising administering to the patient an therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human CD11a.

15 In another embodiment, the invention provides a method of treating a LFA-1-mediated disorder in a human, comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human CD11a. In addition, the invention contemplates the use of the foregoing conjugate in place of full length anti-CD11a antibody in the treatment of an LFA-1-mediated disorder, such as psoriasis and graft rejection, in  
20 a human patient as described in U.S. Pat. No. 5,622,700. In another aspect, the invention contemplates the use of the foregoing conjugate in place of full length anti-CD11a antibody in the treatment of LFA-1-mediated disorders in a human patient as described in WO 98/23761.

(vi) IgE-Mediated Disorders

25 In one embodiment, the invention provides a method for treating an IgE-mediated disorder in a human patient with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human IgE. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of IgE-mediated disorders, including

pathologies characterized by the overproduction and/or hypersensitivity to the immunoglobulin IgE, in a manner similar to the application of anti-IgE antibodies in the treatment of such disease indications that is known in the art, which treatment indications include allergic diseases, such as allergic asthma and allergic rhinitis.

5 In one embodiment, the invention provides a method of treating an IgE-mediated disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human IgE. In another embodiment, the IgE-mediated disorder is an allergic disease. In yet another embodiment, the IgE-mediated  
10 disorder is allergic asthma. In still another embodiment, the IgE-mediated disorder is allergic rhinitis.

In a further embodiment, the invention provides a method of treating an IgE-mediated disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in  
15 the conjugate comprises an antigen binding site that competes with human Fc $\epsilon$ RI for binding to human IgE. In yet another embodiment, the invention provides a method of treating an IgE-mediated disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to membrane-bound IgE  
20 on the surface of human B-lymphocytes but does not bind to soluble IgE bound to Fc $\epsilon$ RI receptor on the surface of human basophils. In addition, the invention contemplates the use of any of the foregoing conjugates in place of full length anti-human IgE antibody in the treatment of an IgE-mediated disorder, such as allergic diseases including allergic asthma and allergic rhinitis, in a human patient as described in International Application No. PCT/US98/13410 (filed June 30,  
25 1998). In another aspect, the invention contemplates the use of any of the foregoing conjgates in place of full length anti-human IgE antibody in the treatment of allergic asthma in a human patient as described in WO 97/04807 (published February 13, 1997) (International Application No. PCT/US96/12275 filed July 24, 1996).

In another embodiment, the invention provides a method of treating an allergic disease in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that competes with human  $\text{Fc}_\epsilon\text{RI}$  for binding to human IgE. In yet another embodiment, the invention provides a method of treating an allergic disease in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to membrane-bound IgE on the surface of human B-lymphocytes but does not bind to soluble IgE bound to  $\text{Fc}_\epsilon\text{RI}$  receptor on the surface of human basophils.

In another embodiment, the invention provides a method of treating allergic asthma in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that competes with human  $\text{Fc}_\epsilon\text{RI}$  for binding to human IgE. In yet another embodiment, the invention provides a method of treating allergic asthma in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to membrane-bound IgE on the surface of human B-lymphocytes but does not bind to soluble IgE bound to  $\text{Fc}_\epsilon\text{RI}$  receptor on the surface of human basophils.

In another embodiment, the invention provides a method of treating allergic rhinitis in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that competes with human  $\text{Fc}_\epsilon\text{RI}$  for binding to human IgE. In yet another embodiment, the invention provides a method of treating allergic rhinitis in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to membrane-bound IgE on the surface of human B-

lymphocytes but does not bind to soluble IgE bound to Fc $\epsilon$ RI receptor on the surface of human basophils.

(vii) Disorders Mediated by Cells Expressing Apo-2 Receptor

In one embodiment, the invention provides a method for treating a human disease mediated by cells expressing Apo-2 receptor with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human Apo-2 receptor. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of Apo-2 receptor-expressing cell-mediated disorders, including cancers susceptible to Apo-2 receptor-mediated apoptosis, in a manner similar to the application of full length anti-Apo-2 receptor antibodies in the treatment of such disease indications that is known in the art, which treatment indications include cancers.

In one embodiment, the invention provides a method of treating a proliferative disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human Apo-2 receptor. The proliferative disorder can be a benign or malignant tumor characterized by cells expressing the Apo-2 receptor, *e.g.* a cancer, such as breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

In another embodiment, the invention provides a method of treating a proliferative disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to the human Apo-2 receptor, and wherein the conjugate is an agonist of the human Apo-2 receptor, *i.e.* capable of inducing Apo-2 receptor-mediated cell apoptosis. The proliferative disorder can be a benign or malignant tumor characterized by cells expressing the Apo-2 receptor, *e.g.* a cancer, such as breast cancer,

squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. In addition, the invention contemplates the use of the foregoing conjugate in place of full length anti-Apo-2 receptor agonist antibody in the treatment of cancers, e.g. colon cancer, as described in WO 98/51793 (published November 19, 1998) (International Application No. PCT/US98/09704 filed May 14, 1998).

(vi) TNF- $\alpha$ -Mediated Disorders

In one embodiment, the invention provides a method for treating a TNF- $\alpha$ -mediated disease with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human TNF- $\alpha$ . Such conjugates have prophylactic and therapeutic applications in a broad spectrum of TNF- $\alpha$ -mediated disorders, including inflammatory disorders and immune disorders, in a manner similar to the application of full length anti-human TNF- $\alpha$  antibodies in the treatment of such disease indications that is known in the art, which treatment indications include Crohn's disease, inflammatory bowel disease, and rheumatoid arthritis.

In one embodiment, the invention provides a method of treating a TNF- $\alpha$ -mediated disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human TNF- $\alpha$ .

In one embodiment, the invention provides a method of treating an inflammatory disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human TNF- $\alpha$ . In another embodiment, the inflammatory disorder is Crohn's disease. In yet another embodiment, the inflammatory disorder is inflammatory bowel disease. In still another embodiment, the inflammatory disorder is rheumatoid arthritis. In addition, the invention contemplates the use of the foregoing conjugate in place of full length anti-human TNF- $\alpha$  antibody in the treatment of

TNF- $\alpha$ -mediated disorders, including inflammatory disorders and immune disorders such as graft-versus-host disease (GHVD) as described in U.S. Pat. No. 5,672,347 (issued September 30, 1997). In another aspect, the invention contemplates the use of the foregoing conjugate in place of full length anti-human TNF- $\alpha$  antibody in the treatment of Crohn's disease as described in U.S. Pat. No. 5,656,272 (issued August 12, 1997). In yet another aspect, the invention contemplates the use of the foregoing conjugate in place of full length anti-human TNF- $\alpha$  antibody in the treatment of rheumatoid arthritis as described in U.S. Pat. No. 5,698,195 (issued December 16, 1997).

(vii) Tissue Factor-Mediated Disorders

In one embodiment, the invention provides a method for treating a tissue factor-mediated disease with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human tissue factor. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of tissue factor-mediated disorders, including pathologies supported by blood coagulation, in a manner similar to the application of full length anti-human tissue factor antibodies in the treatment of such disease indications that is known in the art, which treatment indications include deep vein thrombosis and arterial thrombosis.

In one embodiment, the invention provides a method of treating a tissue factor-mediated disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human tissue factor.

In one embodiment, the invention provides a method of treating a thrombotic or prothrombotic disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human tissue factor. The thrombotic or prothrombotic disorder can be any disorder characteristically associated with a hyperthrombotic state leading to intravascular thrombi or emboli, including diseases involving vascular narrowing or occlusion, such as deep vein thrombosis, arterial thrombosis, atherosclerosis, vascular stenosis, myocardial ischemic diseases including acute

myocardial infarction, reocclusion following angioplasty or atherectomy or thrombolytic treatment for acute myocardial infarction, angina, cerebral ischemic diseases including stroke, venous thrombophlebitis, and pulmonary embolism. In yet another aspect, the invention contemplates the use of the foregoing conjugate in place of full length anti-human tissue factor antibody in the treatment of thrombotic and prothrombotic diseases, such as coronary artery thrombotic diseases as described in European Patent No 0 420 937 B1 (granted November 19, 1994).

In another embodiment, the invention provides a method of inhibiting blood coagulation in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human tissue factor.

(viii) Disorders Mediated by  $\alpha_4\beta_7$  Integrin-Expressing Cells

In one embodiment, the invention provides a method for treating a human disease mediated by an  $\alpha_4\beta_7$  integrin-expressing cell with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human  $\alpha_4\beta_7$  integrin. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of  $\alpha_4\beta_7$  integrin-expressing cell-mediated disorders, including pathologies supported by leukocyte adhesion, in a manner similar to the application of full length anti- $\alpha_4\beta_7$  integrin antibodies in the treatment of such disease indications that is known in the art, which treatment indications include inflammatory bowel disease.

In one embodiment, the invention provides a method of treating a disorder in a human patient mediated by an  $\alpha_4\beta_7$  integrin-expressing cell, comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human  $\alpha_4\beta_7$  integrin.

In another embodiment, the invention provides a method of treating an inflammatory disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in



the conjugate comprises an antigen binding site that binds to human  $\alpha_4\beta_7$  integrin. In another embodiment, the inflammatory disorder is inflammatory bowel disease. In another aspect, the invention contemplates the use of the foregoing conjugate in place of full length anti-human  $\alpha_4\beta_7$  integrin antibody in the treatment of inflammatory disorders in a human patient as described in WO 98/06248 (published February 19, 1998) (International Patent Application No. PCT/US97/13884 filed August 6, 1997).

(ix) GPIIb-IIIa Integrin-Expressing Cell-Mediated Disorders

In one embodiment, the invention provides a method for treating a human disease mediated by a GPIIb-IIIa integrin-expressing cell with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human GPIIb-IIIa integrin. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of GPIIa-IIIb integrin-expressing cell-mediated disorders, including pathologies supported by platelet aggregation, such as thrombotic disorders and prothrombotic disorders, in a manner similar to the application of full length anti-human GPIIb-IIIa integrin antibodies in the treatment of such disease indications that is known in the art, which treatment indications include unstable angina and reocclusion following angioplasty or thrombolytic treatment of acute myocardial infarction.

In one embodiment, the invention provides a method of treating a thrombotic or prothrombotic disorder in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human GPIIb-IIIa integrin. The thrombotic or prothrombotic disorder can be any disorder characteristically associated with a hyperthrombotic state leading to intravascular thrombi or emboli, including diseases involving vascular narrowing or occlusion, such as deep vein thrombosis, arterial thrombosis, atherosclerosis, vascular stenosis, myocardial ischemic diseases including acute myocardial infarction, reocclusion following angioplasty or atherectomy or thrombolytic treatment for acute myocardial infarction, angina, cerebral ischemic diseases including stroke, venous thrombophlebitis, and pulmonary embolism. In another aspect, the invention contemplates the use of the foregoing conjugate in place of full-length anti-human

GPIIb-IIIa antibody in a method for inhibition of thrombus formation in a human patient as described in U.S. Pat. No. 5,387,413 (issued February 7, 1995). In yet another aspect, the invention contemplates the use of the foregoing conjugate in place of unpegylated anti-human GPIIb-IIIa antibody fragment, e.g. Fab, Fab' or F(ab')<sub>2</sub>, in the treatment of thrombotic and prothrombotic diseases, including coronary artery thrombotic diseases such as restenosis following percutaneous coronary artery transluminal angioplasty or atherectomy as described for REOPRO®abciximab in Physician's Desk Reference, 52<sup>nd</sup> Edition (1998), pp. 1498-1501.

In another embodiment, the invention provides a method of inhibiting blood coagulation in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human GPIIb-IIIa integrin.

In still another embodiment, the invention provides a method of inhibiting platelet aggregation in a human patient comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human GPIIb-IIIa integrin.

(x) Disorders Mediated by EGFR-Expressing Cells

In one embodiment, the invention provides a method for treating a human disease mediated by EGFR-expressing cells with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human EGFR. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of EGFR-expressing cell-mediated disorders, including pathologies supported by the proliferation of cells expressing EGFR, such as cancers characterized by overexpression of EGFR, in a manner similar to the application of full length anti-EGFR antibodies in the treatment of such disease indications that is known in the art, which treatment indications include EGFR-overexpressing cancers of the breast, ovary, head and neck, brain, bladder, pancreas, and lung.

In one embodiment, the invention provides a method of treating a cell proliferation disorder in a human patient characterized by overexpression of epidermal growth factor receptor (EGFR) comprising administering to the patient a therapeutically effective amount of any

conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human EGFR. The disorder can be a benign or malignant tumor characterized by the overexpression of the EGFR, *e.g.* a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. In addition, the invention contemplates the use of the foregoing conjugate in place of full length anti-EGFR antibody in the treatment of EGFR-overexpressing cancers as described in WO 96/40210 (published December 19, 1996) (International Application No. PCT/US96/9847 filed June 7, 1996).

(xi) Disorders Mediated by CD3-Expressing Cells

In one embodiment, the invention provides a method for treating a human disease mediated by CD3-expressing cells with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human CD3. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of CD3-expressing cell-mediated disorders, including pathologies supported by the proliferation or activation of cells expressing CD3, such as immune disorders mediated by T-lymphocytes, in a manner similar to the application of full length anti-human CD3 antibodies in the treatment of such disease indications that is known in the art, which treatment indications include graft rejection in transplant recipients.

In one embodiment, the invention provides a method of treating a disorder in a human patient mediated by a CD3-expressing cell, comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human CD3. In another embodiment, the CD3-expressing cell-mediated disorder is characterized by the activation or proliferation of T-lymphocytes, including immune disorders such as graft rejection in transplant recipients. In addition, the invention contemplates the use of the foregoing conjugate in place of full length anti-human CD3 antibody in the treatment of T-lymphocyte

mediated disorders as described in U.S. Pat. No. 4,515,893 (issued May 7, 1985). In another aspect, the invention contemplates the use of the foregoing conjugate in place of full length anti-human CD3 antibody in the treatment of acute allograft rejection in renal transplant recipients as described for ORTHOCLONE OKT3®muromonab-CD3 in Physician's Desk Reference, 52<sup>nd</sup>

5 Edition (1998), pp. 1971-1974.

(xii) Disorders Mediated by TAC-Expressing Cells

In one embodiment, the invention provides a method for treating a human disease mediated by interleukin-2 receptor  $\alpha$ -chain (TAC)-expressing cells with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to human TAC.

10 Such conjugates have prophylactic and therapeutic applications in a broad spectrum of TAC-expressing cell-mediated disorders, including pathologies supported by the proliferation or activation of cells expressing TAC, such as immune disorders mediated by T-lymphocytes or B-lymphocytes, in a manner similar to the application of full length anti-human TAC antibodies in the treatment of such disease indications that is known in the art, which treatment indications  
15 include graft rejection in transplant recipients.

In one embodiment, the invention provides a method of treating a disorder in a human patient mediated by a TAC-expressing cell, comprising administering to the patient a therapeutically effective amount of any conjugate described in this Section (II) wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human TAC. In another embodiment, the TAC-expressing cell-mediated disorder is characterized by the activation or proliferation of T-lymphocytes or B-lymphocytes, including immune disorders such as graft rejection in transplant recipients. In addition, the invention contemplates the use of the foregoing conjugate in place of full length anti-human TAC antibody in the treatment of T-lymphocyte or B-lymphocyte mediated disorders, including graft-versus-host disease (GVHD),  
20 graft rejection in transplant recipients, such as acute graft rejection in renal transplant recipients, and autoimmune diseases such as Type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and myasthenia gravis, as described in U.S. Pat. No. 5,693,761.

(xiii) IL-8-Mediated Disorders

In one embodiment, the invention provides a method for treating an IL-8-mediated disease with any of the conjugates described in this Section (II) that is derived from a parental antibody that binds to rabbit or human IL-8. For example, a conjugate derived from an anti-IL-8 antibody or fragment is useful in the treatment of inflammatory disorders as described in Section (II)(5)(B) below. Such conjugates have prophylactic and therapeutic applications in a broad spectrum of IL-8 mediated diseases, such as inflammatory diseases and asthma, in a manner similar to the widespread efficacy of anti-IL-8 antibodies in the treatment of such disease indications that is known in the art, which treatment indications include: (1) ischemic reperfusion injury of the lung (Sekido et al., Nature, 365: 654 (1993)); (2) acute lung injury and ARDS (WO 96/22785 published August 1, 1996; Folkesson et al., J. Clin. Invest., 96: 107-116 (1995); Mulligan et al., J. Immunol., 150: 5585-5595 (1993)); (3) hypovolemic shock (Hebert, C., “Humanized Anti-IL-8: Potential Therapy for Shock and ARDS”, seminar presented at Keystone Conference on The Role of Cytokines in Leukocyte Trafficking and Disease, held at Copper Mountain Resort, CO, March 31-April 5, 1997; Sharar, S.A., Harlan, J.H., Patterson, C.A., Hebert, C.A., and Winn, R.K., “Reperfusion Injury After Hemorrhagic Shock in Rabbits is Reduced Similarly by IL-8 or CD-18 Monoclonal Antibodies”, manuscript submitted 1998); (4) myocardial infarction (WO 97/40215 published October 30, 1997); (5) cerebral reperfusion injury (Matsumoto et al., Laboratory Invest., 77: 119-125 (1997)); (6) bacterial pneumonia (U.S. Pat. Nos. 5,702,946, 5,677,426, 5,707,622, and 5,686,070); (7) ulcerative colitis (U.S. Pat. Nos. 5,702,946, 5,677,426, 5,707,622, and 5,686,070); and asthma (WO 97/01354 published January 16, 1997).

As shown in the Examples below, the conjugates of the invention mimic the in vitro activities of full-length anti-IL-8 monoclonal antibody (e.g. inhibition of IL-8 binding and activation of human neutrophils as shown in Figs. 54A-54C, 55A-55C and 56A-56C and in Example V below), approximate the in vivo pharmacokinetics (e.g. serum half-life, clearance rate and mean residence time as shown in Fig. 65 and in Example X below) and the in vivo therapeutic efficacy (e.g. the treatment of acute lung injury and ARDS as shown in Figs. 70A-

70E and in Example Z below and the treatment of ischemic reperfusion injury as shown in Fig. 71 and in Example AA below) of full length anti-IL-8 monoclonal antibody. Since conjugates of the invention derived from anti-IL-8 antibodies and fragments display the same or substantially similar in vitro and in vivo activities as full length anti-IL-8 monoclonal antibody across a range of different parameters, including pharmacokinetic characteristics and therapeutic endpoints in various animal models, the data support the efficacy of the conjugates in the same broad spectrum of disease indications that responds to full length anti-IL-8 antibody treatment.

As noted above, any conjugate of the invention derived from an anti-IL-8 antibody or fragment can be advantageously utilized in a method of treating an IL-8 mediated disease or disorder, such as inflammatory diseases. In one embodiment, the invention provides a method of treating an inflammatory disorder in a mammal comprising administering to the mammal an effective amount of a conjugate selected from the group consisting of: (1) every conjugate described in Section (II)(1) above formed by its component parts, i.e. the antibody fragment or fragments and the nonproteinaceous polymer or polymer molecules that form the conjugate, without any extraneous matter in the covalent molecular structure of the conjugate, (2) every conjugate described in Section (II)(1) above modified to contain one or more additional components, in addition to the antibody fragment component(s) and polymer component(s) that form the conjugate, wherein the modification does not alter the essential functional property of the conjugate of substantially improved serum half-life, MRT and/or serum clearance rate as compared to that of the parental antibody fragment from which the conjugate is derived, (3) every conjugate described in Section (II)(1) above modified to incorporate one or more nonproteinaceous labels or reporter molecules, and (4) every conjugate described in Section (II)(1) above modified to incorporate one or more radiolabels; wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human IL-8.

In another aspect, the invention encompasses the foregoing method of treating inflammatory disorders wherein at least one antibody fragment in the conjugate is selected from the group consisting of: (1) an antibody fragment comprising 6G4.2.5LV/L1N35A as defined below; (2) an antibody fragment comprising 6G4.2.5LV/L1N35E as defined below; (3) an

antibody fragment comprising 6G4.2.5HV11 as defined below; (4) an antibody fragment comprising hu6G4.2.5LV/L1N35A as defined below; (5) an antibody fragment comprising hu6G4.2.5LV/L1N35E as defined below; (6) an antibody fragment comprising 6G4.2.5HV as defined below; (7) an antibody fragment comprising 6G4.2.5LV/L1N35A and further comprising the CDRs of 6G4.2.5HV11 as defined below; (8) an antibody fragment comprising 6G4.2.5LV/L1N35E and further comprising the CDRs of 6G4.2.5HV11 as defined below; (9) an antibody fragment comprising hu6G4.2.5LV/L1N35A and further comprising hu6G4.2.5HV as defined below; (10) an antibody fragment comprising hu6G4.2.5LV/L1N35E and further comprising hu6G4.2.5HV as defined below; (11) an antibody fragment comprising 6G4.2.5LV11N35A as defined below; (12) an antibody fragment comprising 6G4.2.5LV11N35E as defined below; (13) an antibody fragment comprising 6G4.2.5LV11N35A and further comprising 6G4.2.5HV11 as defined below; and (14) an antibody fragment comprising 6G4.2.5LV11N35E and further comprising 6G4.2.5HV11 as defined below.

In yet another aspect, the invention encompasses any of the foregoing methods of treating an inflammatory disorder wherein the conjugate contains no more than one antibody fragment, wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH, wherein the antibody fragment is covalently attached to no more than one nonproteinaceous polymer molecule, and wherein the nonproteinaceous polymer molecule is a linear polyethylene glycol having a molecular weight of at least at or about 20 kD, or at least at or about 30 kD or at least at or about 40 kD, or is a branched polyethylene glycol having a molecular weight of at least at or about 40 kD.

In another embodiment, the invention provides a method of treating ischemic reperfusion injury in a mammal comprising administering to the mammal an effective amount of a conjugate selected from the group consisting of: (1) every conjugate described in Section (II)(1) above formed by its component parts, i.e. the antibody fragment or fragments and the nonproteinaceous polymer or polymer molecules that form the conjugate, without any extraneous matter in the covalent molecular structure of the conjugate, (2) every conjugate described in Section (II)(1) above modified to contain one or more additional components, in addition to the antibody

fragment component(s) and polymer component(s) that form the conjugate, wherein the modification does not alter the essential functional property of the conjugate of substantially improved serum half-life, MRT and/or serum clearance rate as compared to that of the parental antibody fragment from which the conjugate is derived, (3) every conjugate described in Section (II)(1) above modified to incorporate one or more nonproteinaceous labels or reporter molecules, and (4) every conjugate described in Section (II)(1) above modified to incorporate one or more radiolabels; wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human IL-8.

In another aspect, the invention encompasses the foregoing method of treating ischemic reperfusion injury wherein at least one antibody fragment in the conjugate is selected from the group consisting of: (1) an antibody fragment comprising 6G4.2.5LV/L1N35A as defined below; (2) an antibody fragment comprising 6G4.2.5LV/L1N35E as defined below; (3) an antibody fragment comprising 6G4.2.5HV11 as defined below; (4) an antibody fragment comprising hu6G4.2.5LV/L1N35A as defined below; (5) an antibody fragment comprising hu6G4.2.5LV/L1N35E as defined below; (6) an antibody fragment comprising hu6G4.2.5HV as defined below; (7) an antibody fragment comprising 6G4.2.5LV/L1N35A and further comprising the CDRs of 6G4.2.5HV11 as defined below; (8) an antibody fragment comprising 6G4.2.5LV/L1N35E and further comprising the CDRs of 6G4.2.5HV11 as defined below; (9) an antibody fragment comprising hu6G4.2.5LV/L1N35A and further comprising hu6G4.2.5HV as defined below; (10) an antibody fragment comprising hu6G4.2.5LV/L1N35E and further comprising hu6G4.2.5HV as defined below; (11) an antibody fragment comprising 6G4.2.5LV11N35A as defined below; (12) an antibody fragment comprising 6G4.2.5LV11N35E as defined below; (13) an antibody fragment comprising 6G4.2.5LV11N35A and further comprising 6G4.2.5HV11 as defined below; and (14) an antibody fragment comprising 6G4.2.5LV11N35E and further comprising 6G4.2.5HV11 as defined below.

In yet another aspect, the invention encompasses the foregoing methods of treating ischemic reperfusion injury wherein the ischemic reperfusion injury is induced by or incident to a surgical procedure, i.e. a surgical tissue reperfusion injury.



In still another aspect, the invention encompasses the foregoing methods of treating ischemic reperfusion injury wherein the ischemic reperfusion injury is a myocardial ischemic reperfusion injury, such as myocardial infarction, reperfusion after cardiac surgery, cardiac arrest, and constriction after percutaneous transluminal coronary angioplasty.

5 In yet another aspect, the invention encompasses any of the foregoing methods of treating ischemic reperfusion injury wherein the conjugate contains no more than one antibody fragment, wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH, wherein the antibody fragment is covalently attached to no more than one nonproteinaceous polymer molecule, and wherein the nonproteinaceous polymer molecule is a linear polyethylene glycol having a molecular weight of at least at or about 20 kD, or at least at or about 30 kD or at  
10 least at or about 40 kD, or is a branched polyethylene glycol having a molecular weight of at least at or about 40 kD.

In another embodiment, the invention provides a method of treating acute lung injury in a mammal comprising administering to the mammal an effective amount of a conjugate selected from the group consisting of: (1) every conjugate described in Section (II)(1) above formed by its component parts, i.e. the antibody fragment or fragments and the nonproteinaceous polymer or polymer molecules that form the conjugate, without any extraneous matter in the covalent molecular structure of the conjugate, (2) every conjugate described in Section (II)(1) above modified to contain one or more additional components, in addition to the antibody fragment component(s) and polymer component(s) that form the conjugate, wherein the modification does  
15 not alter the essential functional property of the conjugate of substantially improved serum half-life, MRT and/or serum clearance rate as compared to that of the parental antibody fragment from which the conjugate is derived, (3) every conjugate described in Section (II)(1) above modified to incorporate one or more nonproteinaceous labels or reporter molecules, and (4) every  
20 conjugate described in Section (II)(1) above modified to incorporate one or more radiolabels; wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human IL-8.

In another aspect, the invention encompasses the foregoing method of treating acute lung

injury wherein at least one antibody fragment in the conjugate is selected from the group consisting of: (1) an antibody fragment comprising 6G4.2.5LV/L1N35A as defined below; (2) an antibody fragment comprising 6G4.2.5LV/L1N35E as defined below; (3) an antibody fragment comprising 6G4.2.5HV11 as defined below; (4) an antibody fragment comprising  
5 hu6G4.2.5LV/L1N35A as defined below; (5) an antibody fragment comprising hu6G4.2.5LV/L1N35E as defined below; (6) an antibody fragment comprising hu6G4.2.5HV as defined below; (7) an antibody fragment comprising 6G4.2.5LV/L1N35A and further comprising the CDRs of 6G4.2.5HV11 as defined below; (8) an antibody fragment comprising 6G4.2.5LV/L1N35E and further comprising the CDRs of 6G4.2.5HV11 as defined below; (9) an  
10 antibody fragment comprising hu6G4.2.5LV/L1N35A and further comprising hu6G4.2.5HV as defined below; (10) an antibody fragment comprising hu6G4.2.5LV/L1N35E and further comprising hu6G4.2.5HV as defined below; (11) an antibody fragment comprising 6G4.2.5LV11N35A as defined below; (12) an antibody fragment comprising 6G4.2.5LV11N35E as defined below; (13) an antibody fragment comprising 6G4.2.5LV11N35A and further comprising 6G4.2.5HV11 as defined below; and (14) an antibody fragment comprising 6G4.2.5LV11N35E and further comprising 6G4.2.5HV11 as defined below.

In yet another aspect, the invention encompasses the foregoing methods of treating acute lung injury wherein the acute lung injury includes adult respiratory distress syndrome (ARDS).

In a further aspect, the invention encompasses any of the foregoing methods of treating  
20 acute lung injury wherein the conjugate contains no more than one antibody fragment, wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH, wherein the antibody fragment is covalently attached to no more than one nonproteinaceous polymer molecule, and wherein the nonproteinaceous polymer molecule is a linear polyethylene glycol having a molecular weight of at least at or about 20 kD, or at least at or about 30 kD or at least at  
25 or about 40 kD.

In a further aspect, the invention encompasses any of the foregoing methods of treating acute lung injury, wherein the patient is selected for prophylactic treatment prior to onset of acute lung injury (with or without progression to ARDS), such as at least 2 hours prior to onset, or at

least 90 minutes prior to onset, or at least 60 minutes prior to onset, or at least 30 minutes prior to onset, by the assessment of biological parameters displayed in the patient's condition that indicate likely progression of disease to acute lung injury which may include ARDS, e.g. by using any of the prognostic methods described in Section (II)(5)(B) below, wherein the conjugate contains no more than one antibody fragment, wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH, wherein the antibody fragment is covalently attached to no more than one nonproteinaceous polymer molecule, and wherein the nonproteinaceous polymer molecule is a linear polyethylene glycol having a molecular weight of at least at or about 20 kD, or at least at or about 30 kD or at least at or about 40 kD, or is a branched polyethylene glycol having a molecular weight of at least at or about 40 kD.

In another embodiment, the invention provides a method of treating hypovolemic shock in a mammal comprising administering to the mammal an effective amount of a conjugate selected from the group consisting of: (1) every conjugate described in Section (II)(1) above formed by its component parts, i.e. the antibody fragment or fragments and the nonproteinaceous polymer or polymer molecules that form the conjugate, without any extraneous matter in the covalent molecular structure of the conjugate, (2) every conjugate described in Section (II)(1) above modified to contain one or more additional components, in addition to the antibody fragment component(s) and polymer component(s) that form the conjugate, wherein the modification does not alter the essential functional property of the conjugate of substantially improved serum half-life, MRT and/or serum clearance rate as compared to that of the parental antibody fragment from which the conjugate is derived, (3) every conjugate described in Section (II)(1) above modified to incorporate one or more nonproteinaceous labels or reporter molecules, and (4) every conjugate described in Section (II)(1) above modified to incorporate one or more radiolabels; wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human IL-8.

In another aspect, the invention encompasses the foregoing method of treating hypovolemic shock wherein at least one antibody fragment in the conjugate is selected from the group consisting of: (1) an antibody fragment comprising 6G4.2.5LV/L1N35A as defined below;

(2) an antibody fragment comprising 6G4.2.5LV/L1N35E as defined below; (3) an antibody fragment comprising 6G4.2.5HV11 as defined below; (4) an antibody fragment comprising hu6G4.2.5LV/L1N35A as defined below; (5) an antibody fragment comprising hu6G4.2.5LV/L1N35E as defined below; (6) an antibody fragment comprising hu6G4.2.5HV as defined below; (7) an antibody fragment comprising 6G4.2.5LV/L1N35A and further comprising the CDRs of 6G4.2.5HV11 as defined below; (8) an antibody fragment comprising 6G4.2.5LV/L1N35E and further comprising the CDRs of 6G4.2.5HV11 as defined below; (9) an antibody fragment comprising hu6G4.2.5LV/L1N35A and further comprising hu6G4.2.5HV as defined below; (10) an antibody fragment comprising hu6G4.2.5LV/L1N35E and further comprising hu6G4.2.5HV as defined below; (11) an antibody fragment comprising 6G4.2.5LV11N35A as defined below; (12) an antibody fragment comprising 6G4.2.5LV11N35E as defined below; (13) an antibody fragment comprising 6G4.2.5LV11N35A and further comprising 6G4.2.5HV11 as defined below; and (14) an antibody fragment comprising 6G4.2.5LV11N35E and further comprising 6G4.2.5HV11 as defined below.

In yet another aspect, the invention encompasses any of the foregoing methods of treating hypovolemic shock wherein the conjugate contains no more than one antibody fragment, wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH, wherein the antibody fragment is covalently attached to no more than one nonproteinaceous polymer molecule, and wherein the nonproteinaceous polymer molecule is a linear polyethylene glycol having a molecular weight of at least at or about 20 kD, or at least at or about 30 kD or at least at or about 40 kD, or is a branched polyethylene glycol having a molecular weight of at least at or about 40 kD.

In another embodiment, the invention provides a method of treating an inflammatory bowel disease in a mammal comprising administering to the mammal an effective amount of a conjugate selected from the group consisting of: (1) every conjugate described in Section (II)(1) above formed by its component parts, i.e. the antibody fragment or fragments and the nonproteinaceous polymer or polymer molecules that form the conjugate, without any extraneous matter in the covalent molecular structure of the conjugate, (2) every conjugate described in

Section (II)(1) above modified to contain one or more additional components, in addition to the antibody fragment component(s) and polymer component(s) that form the conjugate, wherein the modification does not alter the essential functional property of the conjugate of substantially improved serum half-life, MRT and/or serum clearance rate as compared to that of the parental antibody fragment from which the conjugate is derived, (3) every conjugate described in Section (II)(1) above modified to incorporate one or more nonproteinaceous labels or reporter molecules, and (4) every conjugate described in Section (II)(1) above modified to incorporate one or more radiolabels; wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human IL-8.

In another aspect, the invention encompasses the foregoing method of treating an inflammatory bowel disease wherein at least one antibody fragment in the conjugate is selected from the group consisting of: (1) an antibody fragment comprising 6G4.2.5LV/L1N35A as defined below; (2) an antibody fragment comprising 6G4.2.5LV/L1N35E as defined below; (3) an antibody fragment comprising 6G4.2.5HV11 as defined below; (4) an antibody fragment comprising hu6G4.2.5LV/L1N35A as defined below; (5) an antibody fragment comprising hu6G4.2.5LV/L1N35E as defined below; (6) an antibody fragment comprising hu6G4.2.5HV as defined below; (7) an antibody fragment comprising 6G4.2.5LV/L1N35A and further comprising the CDRs of 6G4.2.5HV11 as defined below; (8) an antibody fragment comprising 6G4.2.5LV/L1N35E and further comprising the CDRs of 6G4.2.5HV11 as defined below; (9) an antibody fragment comprising hu6G4.2.5LV/L1N35A and further comprising hu6G4.2.5HV as defined below; (10) an antibody fragment comprising hu6G4.2.5LV/L1N35E and further comprising hu6G4.2.5HV as defined below; (11) an antibody fragment comprising 6G4.2.5LV11N35A as defined below; (12) an antibody fragment comprising 6G4.2.5LV11N35E as defined below; (13) an antibody fragment comprising 6G4.2.5LV11N35A and further comprising 6G4.2.5HV11 as defined below; and (14) an antibody fragment comprising 6G4.2.5LV11N35E and further comprising 6G4.2.5HV11 as defined below.

In still another aspect, the invention encompasses the foregoing methods of treating an inflammatory bowel disease wherein the inflammatory bowel disease is ulcerative colitis.

In yet another aspect, the invention encompasses any of the foregoing methods of treating inflammatory bowel disease wherein the conjugate contains no more than one antibody fragment, wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH, wherein the antibody fragment is covalently attached to no more than one nonproteinaceous  
 5 polymer molecule, and wherein the nonproteinaceous polymer molecule is a linear polyethylene glycol having a molecular weight of at least at or about 20 kD, or at least at or about 30 kD or at least at or about 40 kD, or is a branched polyethylene glycol having a molecular weight of at least at or about 40 kD.

In another embodiment, the invention provides a method of treating a bacterial  
 10 pneumonia in a mammal comprising administering to the mammal an effective amount of a conjugate selected from the group consisting of: (1) every conjugate described in Section (II)(1) above formed by its component parts, i.e. the antibody fragment or fragments and the nonproteinaceous polymer or polymer molecules that form the conjugate, without any extraneous matter in the covalent molecular structure of the conjugate, (2) every conjugate described in  
 15 Section (II)(1) above modified to contain one or more additional components, in addition to the antibody fragment component(s) and polymer component(s) that form the conjugate, wherein the modification does not alter the essential functional property of the conjugate of substantially improved serum half-life, MRT and/or serum clearance rate as compared to that of the parental antibody fragment from which the conjugate is derived, (3) every conjugate described in Section  
 20 (II)(1) above modified to incorporate one or more nonproteinaceous labels or reporter molecules, and (4) every conjugate described in Section (II)(1) above modified to incorporate one or more radiolabels; wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human IL-8.

In another aspect, the invention encompasses the foregoing method of treating bacterial  
 25 pneumonia wherein at least one antibody fragment in the conjugate is selected from the group consisting of: (1) an antibody fragment comprising 6G4.2.5LV/L1N35A as defined below; (2) an antibody fragment comprising 6G4.2.5LV/L1N35E as defined below; (3) an antibody fragment comprising 6G4.2.5HV11 as defined below; (4) an antibody fragment comprising

hu6G4.2.5LV/L1N35A as defined below; (5) an antibody fragment comprising  
 hu6G4.2.5LV/L1N35E as defined below; (6) an antibody fragment comprising hu6G4.2.5HV as  
 defined below; (7) an antibody fragment comprising 6G4.2.5LV/L1N35A and further comprising  
 the CDRs of 6G4.2.5HV11 as defined below; (8) an antibody fragment comprising  
 5 6G4.2.5LV/L1N35E and further comprising the CDRs of 6G4.2.5HV11 as defined below; (9) an  
 antibody fragment comprising hu6G4.2.5LV/L1N35A and further comprising hu6G4.2.5HV as  
 defined below; (10) an antibody fragment comprising hu6G4.2.5LV/L1N35E and further  
 comprising hu6G4.2.5HV as defined below; (11) an antibody fragment comprising  
 6G4.2.5LV11N35A as defined below; (12) an antibody fragment comprising 6G4.2.5LV11N35E  
 10 as defined below; (13) an antibody fragment comprising 6G4.2.5LV11N35A and further  
 comprising 6G4.2.5HV11 as defined below; and (14) an antibody fragment comprising  
 6G4.2.5LV11N35E and further comprising 6G4.2.5HV11 as defined below.

In yet another aspect, the invention encompasses any of the foregoing methods of treating  
 bacterial pneumonia wherein the conjugate contains no more than one antibody fragment,  
 15 wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH,  
 wherein the antibody fragment is covalently attached to no more than one nonproteinaceous  
 polymer molecule, and wherein the nonproteinaceous polymer molecule is a linear polyethylene  
 glycol having a molecular weight of at least at or about 20 kD, or at least at or about 30 kD or at  
 least at or about 40 kD, or is a branched polyethylene glycol having a molecular weight of at  
 20 least at or about 40 kD.

In another embodiment, the invention provides a method of treating an asthmatic disease  
 in a mammal comprising administering to the mammal an effective amount of a conjugate  
 selected from the group consisting of: (1) every conjugate described in Section (II)(1) above  
 formed by its component parts, i.e. the antibody fragment or fragments and the nonproteinaceous  
 25 polymer or polymer molecules that form the conjugate, without any extraneous matter in the  
 covalent molecular structure of the conjugate, (2) every conjugate described in Section (II)(1)  
 above modified to contain one or more additional components, in addition to the antibody  
 fragment component(s) and polymer component(s) that form the conjugate, wherein the

modification does not alter the essential functional property of the conjugate of substantially improved serum half-life, MRT and/or serum clearance rate as compared to that of the parental antibody fragment from which the conjugate is derived, (3) every conjugate described in Section (II)(1) above modified to incorporate one or more nonproteinaceous labels or reporter molecules, and (4) every conjugate described in Section (II)(1) above modified to incorporate one or more radiolabels; wherein at least one antibody fragment in the conjugate comprises an antigen binding site that binds to human IL-8.

In another aspect, the invention encompasses the foregoing method of treating an asthmatic disease wherein at least one antibody fragment in the conjugate is selected from the group consisting of: (1) an antibody fragment comprising 6G4.2.5LV/L1N35A as defined below; (2) an antibody fragment comprising 6G4.2.5LV/L1N35E as defined below; (3) an antibody fragment comprising 6G4.2.5HV11 as defined below; (4) an antibody fragment comprising hu6G4.2.5LV/L1N35A as defined below; (5) an antibody fragment comprising hu6G4.2.5LV/L1N35E as defined below; (6) an antibody fragment comprising hu6G4.2.5HV as defined below; (7) an antibody fragment comprising 6G4.2.5LV/L1N35A and further comprising the CDRs of 6G4.2.5HV11 as defined below; (8) an antibody fragment comprising 6G4.2.5LV/L1N35E and further comprising the CDRs of 6G4.2.5HV11 as defined below; (9) an antibody fragment comprising hu6G4.2.5LV/L1N35A and further comprising hu6G4.2.5HV as defined below; (10) an antibody fragment comprising hu6G4.2.5LV/L1N35E and further comprising hu6G4.2.5HV as defined below; (11) an antibody fragment comprising 6G4.2.5LV11N35A as defined below; (12) an antibody fragment comprising 6G4.2.5LV11N35E as defined below; (13) an antibody fragment comprising 6G4.2.5LV11N35A and further comprising 6G4.2.5HV11 as defined below; and (14) an antibody fragment comprising 6G4.2.5LV11N35E and further comprising 6G4.2.5HV11 as defined below.

In yet another aspect, the invention encompasses the foregoing methods of treating asthmatic disease wherein the asthmatic disease is allergic asthma.

In yet another aspect, the invention encompasses any of the foregoing methods of treating an asthmatic disease wherein the conjugate contains no more than one antibody fragment,



wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH, wherein the antibody fragment is covalently attached to no more than one nonproteinaceous polymer molecule, and wherein the nonproteinaceous polymer molecule is a linear polyethylene glycol having a molecular weight of at least at or about 20 kD, or at least at or about 30 kD or at least at or about 40 kD, or is a branched polyethylene glycol having a molecular weight of at least at or about 40 kD.

In a preferred embodiment, the invention encompasses any of the foregoing methods of treating inflammatory diseases or asthmatic diseases wherein the mammal is a human.

Therapeutic formulations of the conjugate of the invention can be prepared by utilizing the same procedures described for the formulation of the anti-IL-8 antibodies and fragments of the invention in Section (II)(5)(B) below. The conjugate of the invention can be administered in place of the parent antibody for a given disease indication by modifying the formulation, dosage, administration protocol, and other aspects of a therapeutic regimen as required by the different pharmacodynamic characteristics of the conjugate and as dictated by common medical knowledge and practice.

e. Reagent Uses for Large Effective Size Conjugates

The conjugate of the invention also finds application as a reagent in an animal model system for in vivo study of the biological functions of the antigen recognized by the conjugate. The conjugate would enable the practitioner to inactivate or detect the cognate antigen in circulation or in tissue for a far greater period of time than would be possible with art-known constructs while removing any Fc interaction (which could attend the use of an intact antibody) from the system. In addition, the increased half-life of the conjugate of the invention can be applied advantageously to the induction of tolerance for the underivatized antibody fragment in a test animal by employing the Wie et al., Int. Archs. Allergy Appl. Immunol., 64: 84-99 (1981) method for allergen tolerization, which would permit the practitioner to repeatedly challenge the tolerized animal with the underivatized parental antibody fragment without generating an immune response against the parental fragment.

## 2. HUMANIZED 6G4.2.5 MONOCLONAL ANTIBODIES AND ANTIBODY

### FRAGMENTS

In one embodiment, the invention provides an antibody fragment or full length antibody comprising a heavy chain comprising the amino acid sequence of amino acids 1-230 (herein referred to as "6G4.2.5HV11") of the humanized anti-IL-8 6G4.2.5v11 heavy chain polypeptide amino acid sequence of Figs. 37A-37B (SEQ ID NO: 60).

The invention encompasses a single chain antibody fragment comprising the 6G4.2.5HV11, with or without any additional amino acid sequence. In one embodiment, the invention provides a single chain antibody fragment comprising the 6G4.2.5HV11 without any associated light chain amino acid sequence, i.e. a single chain species that makes up one half of a Fab fragment.

Further provided herein are an antibody or antibody fragment comprising the 6G4.2.5HV11, and further comprising a light chain comprising the amino acid sequence of amino acids 1-219 (herein referred to as "6G4.2.5LV11") of the humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51).

In one embodiment, the invention provides a single chain antibody fragment wherein the 6G4.2.5HV11 and the 6G4.2.5LV11 are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment comprises the 6G4.2.5HV11 joined to the 6G4.2.5LV11 by means of a flexible peptide linker sequence, wherein the heavy chain and light chain domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fab species. In another embodiment, the single chain antibody fragment is a species comprising the 6G4.2.5HV11 joined to the 6G4.2.5LV11 by a linker that is too short to permit intramolecular pairing of complementary domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In yet another embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the 6G4.2.5HV11 and a second polypeptide chain comprises the 6G4.2.5LV11 and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds. In a preferred embodiment, the

foregoing two-chain antibody fragment is selected from the group consisting of Fab, Fab', Fab'-SH, and F(ab')<sub>2</sub>.

The invention also provides an antibody or antibody fragment comprising a heavy chain containing the 6G4.2.5HV11 and optionally further comprising a light chain containing the 6G4.2.5LV11, wherein the heavy chain, and optionally the light chain, is (are) fused to an additional moiety, such as additional immunoglobulin constant domain sequence. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.* (supra).

In a preferred embodiment, the antibody or antibody fragment comprises the 6G4.2.5HV11 in a heavy chain that is fused to or contains a leucine zipper sequence. The leucine zipper can increase the affinity and/or production efficiency of the antibody or antibody fragment of interest. Suitable leucine zipper sequences include the jun and fos leucine zippers taught by Kostelney *et al.*, *J. Immunol.*, 148: 1547-1553 (1992) and the GCN4 leucine zipper described in the Examples below. In a preferred embodiment, the antibody or antibody fragment comprises the 6G4.2.5HV11 fused at its C-terminus to the GCN4 leucine zipper to yield the amino acid sequence of amino acids 1-275 (herein referred to as "6G4.2.5HV11GCN4") of the heavy chain polypeptide amino acid sequence of Figs. 37A-37B (SEQ ID NO: 60).

### 3. VARIANTS OF HUMANIZED 6G4.2.5 MONOCLONAL ANTIBODIES AND ANTIBODY FRAGMENTS

The invention additionally encompasses humanized anti-IL-8 monoclonal antibody and antibody fragments comprising variants of the 6G4.2.5 complementarity determining regions (CDRs) or variants of the 6G4.2.5v11 variable domains which exhibit higher affinity for human IL-8 and/or possess properties that yield greater efficiency in recombinant production processes.

A. 6G4.2.5LV VARIANTS

In one aspect, the invention provides humanized anti-IL-8 monoclonal antibodies and antibody fragments comprising the complementarity determining regions (referred to herein as the "CDRs of 6G4.2.5LV") L1, L2, and L3 of the 6G4.2.5 light chain variable domain amino acid sequence of Fig. 24, wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35).

In addition, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a humanized light chain variable domain comprising a variant (hereinafter referred to a "6G4.2.5LV CDRs variant") of the complementarity determining regions L1, L2, and L3 of the 6G4.2.5 variable light chain domain amino acid sequence of Fig. 24 (SEQ ID NO: 35). In one embodiment, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L1N35X<sub>35</sub>") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that any amino acid other than Asn (denoted as "X<sub>35</sub>") is substituted for Asn at amino acid position 35, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35). In a preferred embodiment, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L1N35A") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that Ala is substituted for Asn at amino acid position 35, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35). In another preferred embodiment, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L1N35E")

wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that Glu is substituted for Asn at amino acid position 35, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35).

5 In a second aspect, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L1S26X<sub>26</sub>") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that any amino acid other than Ser (denoted as "X<sub>26</sub>") is substituted for Ser at amino acid position 26, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35). In a preferred embodiment, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L1S26A") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that Ala is substituted for Ser at amino acid position 26, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35).

15 In a third aspect, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L3H98X<sub>98</sub>") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that any amino acid other than His (denoted as "X<sub>98</sub>") is substituted for His at amino acid position 98. In a preferred embodiment, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L3H98A") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35).

to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that Ala is substituted for His at amino acid position 98.

In a fourth aspect, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L1S26X<sub>26</sub>,N35X<sub>35</sub>") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that any amino acid other than Ser (denoted as "X<sub>26</sub>") is substituted for Ser at amino acid position 26 and any amino acid other than Asn (denoted as "X<sub>35</sub>") is substituted for Asn at amino acid position 35, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO:35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO:35). In a preferred embodiment, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L1S26A,N35A") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO:35) with the proviso that Ala is substituted for Ser at amino acid position 26 and Ala is substituted for Asn at amino acid position 35, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO 35).

In a fifth aspect, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L1N35X<sub>35</sub>/L3H98X<sub>98</sub>") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO 35) with the proviso that any amino acid other than Asn (denoted as "X<sub>35</sub>") is substituted for Asn at amino acid position 35, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO 35) with the proviso that any amino acid other than His (denoted as "X<sub>98</sub>") is substituted for His at amino acid position 98.

In a preferred embodiment, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L1N35A/L3H98A") wherein L1 corresponds to amino acids 24-39 of the amino

acid sequence of Fig. 24 (SEQ ID NO 35) with the proviso that Ala is substituted for Asn at amino acid position 35, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO 35) with the proviso that Ala is substituted for His at amino acid position 98.

In a sixth aspect, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L1S26X<sub>26</sub>/L3H98X<sub>98</sub>") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO 35) with the proviso that any amino acid other than Ser (denoted as "X<sub>26</sub>") is substituted for Ser at amino acid position 26, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO 35) with the proviso that any amino acid other than His (denoted as "X<sub>98</sub>") is substituted for His at amino acid position 98. In a preferred embodiment, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (herein referred to as "6G4.2.5LV/L1S26A/L3H98A") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO 35) with the proviso that Ala is substituted for Ser at amino acid position 26, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that Ala is substituted for His at amino acid position 98.

In a seventh aspect, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (here referred to as "6G4.2.5LV/L1S26X<sub>26</sub>,N35X<sub>35</sub>/L3H98X<sub>98</sub>") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that any amino acid other than Ser (denoted as "X<sub>26</sub>") is substituted for Ser at amino acid position 26 and any amino acid other than Asn (denoted as "X<sub>35</sub>") is substituted for Asn at amino acid position 35, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35)

with the proviso that any amino acid other than His (denoted as "X<sub>98</sub>") is substituted for His at amino acid position 98. In a preferred embodiment, the invention provides a variant 6G4.2.5 humanized antibody or antibody fragment comprising a 6G4.2.5LV CDRs variant (here referred to as "6G4.2.5LV/L1S26A,N35A/L3H98A") wherein L1 corresponds to amino acids 24-39 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that Ala is substituted for Ser at amino acid position 26 and Ala is substituted for Asn at amino acid position 35, L2 corresponds to amino acids 55-61 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35), and L3 corresponds to amino acids 94-102 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35) with the proviso that Ala is substituted for His at amino acid position 98.

The humanized light chain variable domains of the invention can be constructed by using any of the techniques for antibody humanization known in the art. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, Nature 321:522 (1986); Riechmann *et al.*, Nature 332:323 (1988); Verhoeven *et al.*, Science 239:1534 (1988)), by substituting the CDRs of 6G4.2.5LV or the CDRs of a 6G4.2.5LV CDRs variant for the corresponding sequences of a human antibody light chain variable domain. Accordingly, such "humanized" derivatives containing the CDRs of 6G4.2.5LV or the CDRs of a 6G4.2.5LV CDRs variant are chimeric (Cabilly *et al.*, *supra*). The humanized light chain variable domain comprising the CDRs of 6G4.2.5LV or the CDRs of a 6G4.2.5LV CDRs variant can also contain some FR residues that are substituted by residues from analogous sites in the murine 6G4.2.5 antibody light chain variable domain ("6G4.2.5LV"). The complete amino acid sequence of 6G4.2.5LV is set out as amino acids 1-114 of the amino acid sequence of Fig. 24 (SEQ ID NO: 35).

The invention further provides a humanized antibody or antibody fragment comprising a humanized light chain variable domain comprising the CDRs of 6G4.2.5LV or the CDRs of a 6G4.2.5LV CDRs variant as described above, and further comprising a humanized heavy chain variable domain comprising the complementarity determining regions (CDRs) H1, H2, and H3 of the 6G4.2.5 (murine monoclonal antibody) variable heavy chain domain amino acid sequence of Fig. 25 (SEQ ID NO: 37), wherein H1 correspond to amino acids 26-35 of the amino acid



sequence of Fig. 25 (SEQ ID NO: 37), wherein H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and wherein H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37). The above-described H1, H2, and H3 CDRs of the 6G4.2.5 heavy chain variable domain ("6G4.2.5HV") are collectively referred to as the "CDRs of 6G4.2.5HV".

In another embodiment, the invention provides a humanized antibody or antibody fragment comprising a humanized light chain variable domain comprising the CDRs of 6G4.2.5LV or the CDRs of a 6G4.2.5LV CDRs variant as described above, and further comprising a humanized heavy chain variable domain comprising a variant (herein referred to as a "6G4.2.5HV CDRs variant") of the H1, H2, and H3 CDRs of the 6G4.2.5 (murine monoclonal antibody) variable heavy chain domain amino acid sequence of Fig. 25 (SEQ ID NO: 37). In one 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37). In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37).

In a second 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H2S54Z<sub>54</sub>"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37). In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H2S54A"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig.

25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37).

5 In a third 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H3D100E"), wherein H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), wherein H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and wherein H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100.

10 In a fourth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H3R102K"), wherein H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), wherein H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and wherein H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102.

15 In a fifth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H3D106E"), wherein H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), wherein H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and wherein H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 106.

20 In a seventh 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H3D100E,R102K"), wherein H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), wherein H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and wherein H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Lys is substituted for Arg at amino acid

position 102.

In an eighth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H3R102K,D106E"), wherein H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), wherein H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and wherein H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106.

In a ninth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H3D100E,D106E"), wherein H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), wherein H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and wherein H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Glu is substituted for Asp at amino acid position 106.

In a tenth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H3D100E,R102K,D106E"), wherein H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), wherein H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and wherein H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100, Lys is substituted for Arg at amino acid position 102, and Glu is substituted for Asp at amino acid position 106.

In an eleventh 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3

corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37). In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H2S54A"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37).

In a twelfth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H3D100E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100.

In a thirteenth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H3R102K"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H3R102K"), H1 correspond to amino acids 26-35 of

the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102.

A fourteenth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H3D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H3D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 106.

A fifteenth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,R102K"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Lys is substituted for Arg at amino acid position 102. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H3D100E,R102K"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at

amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Lys is substituted for Arg at amino acid position 102.

5 In a sixteenth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H3R102K,D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H3R102K,D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106.

15 In a seventeenth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H3D100E,D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at

amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Glu is substituted for Asp at amino acid position 106.

5 In an eighteenth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,R102K,D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100, Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H3D100E,R102K,D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100, Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106.

15 In a nineteenth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H2S54A/H3D100E"), H1 corresponds to amino acids 26-35 of

the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100.

In a twentieth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H2S54Z<sub>54</sub>/H3R102K"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H2S54A/H3R102K"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102.

In a twenty-first 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H2S54Z<sub>54</sub>/H3D106E"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H2S54A/H3D106E"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for



Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 106.

In a twenty-second 6G4.2.5HV CDRs variant (referred to herein as

5 "6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E,R102K"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Lys is substituted for Arg at amino acid position 102. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H2S54A/H3D100E,R102K"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Lys is substituted for Arg at amino acid position 102.

In a twenty-third 6G4.2.5HV CDRs variant (referred to herein as

20 "6G4.2.5HV/H2S54Z<sub>54</sub>/H3R102K,D106E"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H2S54A/H3R102K,D106E"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at

amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106.

In a twenty-fourth 6G4.2.5HV CDRs variant (referred to herein as

"6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E,D106E"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as

"6G4.2.5HV/H2S54A/H3D100E,D106E"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Glu is substituted for Asp at amino acid position 106.

In a twenty-fifth 6G4.2.5HV CDRs variant (referred to herein as

"6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E,R102K,D106E"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100, Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as

"6G4.2.5HV/H2S54A/H3D100E,R102K,D106E"), H1 corresponds to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37), H2 corresponds to amino acids 50-66 of the

amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100, Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106.

In a twenty-sixth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H2S54A/H3D100E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100.

In a twenty-seventh 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3R102K"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102. In a preferred 6G4.2.5HV

CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H2S54A/H3R102K"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102.

In a twenty-eighth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H2S54A/H3D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 106.

In a twenty-ninth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,R102K"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54,

and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Lys is substituted for Arg at amino acid position 102. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H2S54A/H3D100E,R102K"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Lys is substituted for Arg at amino acid position 102.

In a thirtieth 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3R102K,D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H2S54A/H3R102K,D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106.

In a thirty-first 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,D106E"), H1 correspond to amino acids 26-35 of

the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H2S54A/H3D100E,D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100 and Glu is substituted for Asp at amino acid position 106.

In a thirty-second 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,R102K,D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>31</sub>") is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that any amino acid other than Ser (denoted as "Z<sub>54</sub>") is substituted for Ser at amino acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100, Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106. In a preferred 6G4.2.5HV CDRs variant (referred to herein as "6G4.2.5HV/H1S31A/H2S54A/H3D100E,R102K,D106E"), H1 correspond to amino acids 26-35 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino acid position 31, H2 corresponds to amino acids 50-66 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Ala is substituted for Ser at amino

acid position 54, and H3 corresponds to amino acids 99-111 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37) with the proviso that Glu is substituted for Asp at amino acid position 100, Lys is substituted for Arg at amino acid position 102 and Glu is substituted for Asp at amino acid position 106.

5 As in the humanization of the light chain variable domain described above, a humanized heavy chain variable domain is constructed by substituting the CDRs of 6G4.2.5HV or the CDRs of a 6G4.2.5HV CDRs variant for the corresponding sequences in a human heavy chain variable domain. The humanized heavy chain variable domain comprising the CDRs of 6G4.2.5HV or the CDRs of a 6G4.2.5HV CDRs variant can also contain some FR residues that are substituted  
10 by residues from analogous sites in the murine 6G4.2.5 antibody heavy chain variable domain. The complete amino acid sequence of 6G4.2.5HV is set out as amino acids 1-122 of the amino acid sequence of Fig. 25 (SEQ ID NO: 37).

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies and antibody fragments is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al.*, J. Immunol. 151: 2296 (1993); Chothia and Lesk, J. Mol. Biol. 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains.  
20 The same framework can be used for several different humanized antibodies (Carter *et al.*, Proc. Natl. Acad. Sci. U.S.A. 89:4285 (1992); Presta *et al.*, J. Immunol. 151:2623 (1993)).

It is also important that the antibodies and antibody fragments of the invention be humanized with retention of high affinity for human IL-8 and other favorable biological  
25 properties. To achieve this goal, according to a preferred method, the humanized antibodies and antibody fragments of the invention are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly

available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and parental sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved.

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV are collectively referred to herein as "hu6G4.2.5LV".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1N35X<sub>35</sub> are collectively referred to herein as "hu6G4.2.5LV/L1N35X<sub>35</sub>".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1N35A are collectively referred to herein as "hu6G4.2.5LV/L1N35A".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1N35E are collectively referred to herein as "hu6G4.2.5LV/L1N35E".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1S26X<sub>26</sub> are collectively referred to herein as "hu6G4.2.5LV/L1S26X<sub>26</sub>".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1S26A are collectively referred to herein as "hu6G4.2.5LV/L1S26A".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L3H98X<sub>98</sub> are collectively referred to herein as "hu6G4.2.5LV/L3H98X<sub>98</sub>".



Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L3H98A are collectively referred to herein as "hu6G4.2.5LV/L3H98A".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1S26X<sub>26</sub>,N35X<sub>35</sub> are collectively referred to herein as "hu6G4.2.5LV/L1S26X<sub>26</sub>,N35X<sub>35</sub>".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1S26A,N35A are collectively referred to herein as "hu6G4.2.5LV/L1S26A,N35A".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1N35X<sub>35</sub>/L3H98X<sub>98</sub> are collectively referred to herein as "hu6G4.2.5LV/L1N35X<sub>35</sub>/L3H98X<sub>98</sub>".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1N35A/L3H98A are collectively referred to herein as "hu6G4.2.5LV/L1N35A/L3H98A".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1S26X<sub>26</sub>/L3H98X<sub>98</sub> are collectively referred to herein as "hu6G4.2.5LV/L1S26X<sub>26</sub>/L3H98X<sub>98</sub>".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1S26A/L3H98A are collectively referred to herein as "hu6G4.2.5LV/L1S26A/L3H98A".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1S26X<sub>26</sub>,N35X<sub>35</sub>/L3H98X<sub>98</sub> are collectively referred to herein as "hu6G4.2.5LV/L1S26X<sub>26</sub>,N35X<sub>35</sub>/L3H98X<sub>98</sub>".

Any and all humanized light chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5LV/L1S26A,N35A/L3H98A are collectively referred to herein as "hu6G4.2.5LV/L1S26A,N35A/L3H98A".

The humanized light chain variable domain amino acid sequences of

hu6G4.2.5LV/L1N35X<sub>35</sub>, hu6G4.2.5LV/L1S26X<sub>26</sub>, hu6G4.2.5LV/L1S26X<sub>26</sub>/L3H98X<sub>98</sub>,  
hu6G4.2.5LV/L1S26X<sub>26</sub>,N35X<sub>35</sub>, hu6G4.2.5LV/L1N35X<sub>35</sub>/L3H98X<sub>98</sub>,  
hu6G4.2.5LV/L1S26X<sub>26</sub>/L3H98X<sub>98</sub>, and hu6G4.2.5LV/L1S26X<sub>26</sub>,N35X<sub>35</sub>/L3H98X<sub>98</sub> are  
collectively referred to herein as "hu6G4.2.5LV/vL1-3X".

5           The humanized light chain variable domain amino acid sequences of  
hu6G4.2.5LV/L1N35A, hu6G4.2.5LV/L1S26A, hu6G4.2.5LV/L1S26A/L3H98A,  
hu6G4.2.5LV/L1S26A,N35A, hu6G4.2.5LV/L1N35A/L3H98A,  
hu6G4.2.5LV/L1S26A/L3H98A, hu6G4.2.5LV/L1S26A,N35A/L3H98A are collectively  
referred to herein as "hu6G4.2.5LV/vL1-3A".

10           Any and all humanized heavy chain variable domain amino acid sequences which  
comprise the CDRs of 6G4.2.5HV are collectively referred to herein as "hu6G4.2.5HV".

Any and all humanized heavy chain variable domain amino acid sequences which  
comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub> are collectively referred to herein as  
"hu6G4.2.5HV/H1S31Z<sub>31</sub>".

15           Any and all humanized heavy chain variable domain amino acid sequences which  
comprise the CDRs of 6G4.2.5HV/H1S31A are collectively referred to herein as  
"hu6G4.2.5HV/H1S31A".

Any and all humanized heavy chain variable domain amino acid sequences which  
comprise the CDRs of 6G4.2.5HV/H2S54Z<sub>54</sub> are collectively referred to herein as  
"hu6G4.2.5HV/H2S54Z<sub>54</sub>".

20           Any and all humanized heavy chain variable domain amino acid sequences which  
comprise the CDRs of 6G4.2.5HV/H2S54A are collectively referred to herein as  
"hu6G4.2.5HV/H2S54A".

25           Any and all humanized heavy chain variable domain amino acid sequences which  
comprise the CDRs of 6G4.2.5HV/H3D100E are collectively referred to herein as  
"hu6G4.2.5HV/H3D100E".

Any and all humanized heavy chain variable domain amino acid sequences which  
comprise the CDRs of 6G4.2.5HV/H3R102K are collectively referred to herein as

"hu6G4.2.5HV/H3R102K".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H3D106E are collectively referred to herein as "hu6G4.2.5HV/H3D106E".

5 Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H3D100E,R102K are collectively referred to herein as "hu6G4.2.5HV/H3D100E,R102K".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H3R102K,D106E are collectively referred to herein as  
10 "hu6G4.2.5HV/H3R102K,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H3D100E,D106E are collectively referred to herein as "hu6G4.2.5HV/H3D100E,D106E".

15 Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H3D100E,R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H3D100E,R102K,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub> are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>".

20 Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H3R102K are collectively referred to herein as  
25 "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3R102K".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H3D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,R102K are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,R102K".

Any and all humanized heavy chain variable domain amino acid sequences which  
5 comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H3R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3R102K,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,D106E".

10 Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,R102K,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E are collectively referred to herein as  
15 "hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H2S54Z<sub>54</sub>/H3R102K are collectively referred to herein as  
"hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3R102K".

Any and all humanized heavy chain variable domain amino acid sequences which  
20 comprise the CDRs of 6G4.2.5HV/H2S54Z<sub>54</sub>/H3D106E are collectively referred to herein as "hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H2S54Z<sub>54</sub>/H3R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3R102K,D106E".

25 Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E,D106E are collectively referred to herein as "hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which

comprise the CDRs of 6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E,R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E,R102K,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3R102K are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3R102K".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,R102K are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,R102K".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3R102K,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,R102K,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H2S54A are collectively referred to herein as "hu6G4.2.5HV/H1S31A/H2S54A".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H3D100E are collectively referred to herein as

"hu6G4.2.5HV/H1S31A/H3D100E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H3R102K are collectively referred to herein as "hu6G4.2.5HV/H1S31A/H3R102K".

5 Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H3D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31A/H3D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H3D100E,R102K are collectively referred to herein  
10 as "hu6G4.2.5HV/H1S31A/H3D100E,R102K".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H3R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31A/H3R102K,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which  
15 comprise the CDRs of 6G4.2.5HV/H1S31A/H3D100E,D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31A/H3D100E,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H3D100E,R102K,D106E are collectively referred to  
herein as "hu6G4.2.5HV/H1S31A/H3D100E,R102K,D106E".

20 Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H2S54A/H3D100E are collectively referred to herein as "hu6G4.2.5HV/H2S54A/H3D100E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H2S54A/H3R102K are collectively referred to herein as  
25 "hu6G4.2.5HV/H2S54A/H3R102K".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H2S54A/H3D106E are collectively referred to herein as "hu6G4.2.5HV/H2S54A/H3D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H2S54A/H3R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H2S54A/H3R102K,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which  
5 comprise the CDRs of 6G4.2.5HV/H2S54A/H3D100E,D106E are collectively referred to herein as "hu6G4.2.5HV/H2S54A/H3D100E,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H2S54A/H3D100E,R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H2S54A/H3D100E,R102K,D106E".

10 Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H2S54A/H3D100E are collectively referred to herein as "hu6G4.2.5HV/H1S31A/H2S54A/H3D100E".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H2S54A/H3R102K are collectively referred to  
15 herein as "hu6G4.2.5HV/H1S31A/H2S54A/H3R102K".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H2S54A/H3D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31A/H2S54A/H3D106E".

Any and all humanized heavy chain variable domain amino acid sequences which  
20 comprise the CDRs of 6G4.2.5HV/H1S31A/H2S54A/H3D100E,R102K are collectively referred to herein as "hu6G4.2.5HV/H1S31A/H2S54A/H3D100E,R102K".

Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H2S54A/H3R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31A/H2S54A/H3R102K,D106E".

25 Any and all humanized heavy chain variable domain amino acid sequences which comprise the CDRs of 6G4.2.5HV/H1S31A/H2S54A/H3D100E,D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31A/H2S54A/H3D100E,D106E".

Any and all humanized heavy chain variable domain amino acid sequences which

comprise the CDRs of 6G4.2.5HV/H1S31A/H2S54A/H3D100E,R102K,D106E are collectively referred to herein as "hu6G4.2.5HV/H1S31A/H2S54A/H3D100E,R102K,D106E".

The humanized heavy chain variable domain amino acid sequences of  
 hu6G4.2.5HV/H1S31Z<sub>31</sub>, hu6G4.2.5HV/H2S54Z<sub>54</sub>, hu6G4.2.5HV/H3D100E,  
 5 hu6G4.2.5HV/H3R102K, hu6G4.2.5HV/H3D106E, hu6G4.2.5HV/H3D100E,R102K,  
 hu6G4.2.5HV/H3R102K,D106E, hu6G4.2.5HV/H3D100E,D106E,  
 hu6G4.2.5HV/H3D100E,R102K,D106E, hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>,  
 hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E, hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3R102K,  
 hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3D106E, hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,R102K,  
 10 hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3R102K,D106E, hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,D106E,  
 hu6G4.2.5HV/H1S31Z<sub>31</sub>/H3D100E,R102K,D106E, hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E,  
 hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3R102K, hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3D106E,  
 hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3R102K,D106E, hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E,D106E,  
 hu6G4.2.5HV/H2S54Z<sub>54</sub>/H3D100E,R102K,D106E,  
 5 hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E, hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3R102K,  
 hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D106E,  
 hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,R102K,  
 hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3R102K,D106E,  
 hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,D106E, and  
 20 hu6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>/H3D100E,R102K,D106E are collectively referred to herein  
 as "hu6G4.2.5HV/vH1-3Z".

The humanized heavy chain variable domain amino acid sequences of  
 hu6G4.2.5HV/H1S31A, hu6G4.2.5HV/H2S54A, hu6G4.2.5HV/H3D100E,  
 hu6G4.2.5HV/H3R102K, hu6G4.2.5HV/H3D106E, hu6G4.2.5HV/H3D100E,R102K,  
 25 hu6G4.2.5HV/H3R102K,D106E, hu6G4.2.5HV/H3D100E,D106E,  
 hu6G4.2.5HV/H3D100E,R102K,D106E, hu6G4.2.5HV/H1S31A/H2S54A,  
 hu6G4.2.5HV/H1S31A/H3D100E, hu6G4.2.5HV/H1S31A/H3R102K,  
 hu6G4.2.5HV/H1S31A/H3D106E, hu6G4.2.5HV/H1S31A/H3D100E,R102K,



hu6G4.2.5HV/H1S31A/H3R102K,D106E, hu6G4.2.5HV/H1S31A/H3D100E,D106E,  
 hu6G4.2.5HV/H1S31A/H3D100E,R102K,D106E, hu6G4.2.5HV/H2S54A/H3D100E,  
 hu6G4.2.5HV/H2S54A/H3R102K, hu6G4.2.5HV/H2S54A/H3D106E,  
 hu6G4.2.5HV/H2S54A/H3R102K,D106E, hu6G4.2.5HV/H2S54A/H3D100E,D106E,  
 5 hu6G4.2.5HV/H2S54A/H3D100E,R102K,D106E, hu6G4.2.5HV/H1S31A/H2S54A/H3D100E,  
 hu6G4.2.5HV/H1S31A/H2S54A/H3R102K, hu6G4.2.5HV/H1S31A/H2S54A/H3D106E,  
 hu6G4.2.5HV/H1S31A/H2S54A/H3D100E,R102K,  
 hu6G4.2.5HV/H1S31A/H2S54A/H3R102K,D106E,  
 hu6G4.2.5HV/H1S31A/H2S54A/H3D100E,D106E, and  
 10 hu6G4.2.5HV/H1S31A/H2S54A/H3D100E,R102K,D106E are collectively referred to herein as  
 "hu6G4.2.5HV/vH1-3A".

The invention provides a humanized antibody or antibody fragment that comprises a light  
 chain variable domain comprising the hu6G4.2.5LV/vL1-3X. In another embodiment, the  
 invention provides a humanized antibody or antibody fragment that comprises a light chain  
 15 variable domain comprising the hu6G4.2.5LV/vL1-3A. In yet another embodiment, the  
 invention provides a humanized antibody or antibody fragment that comprises a light chain  
 variable domain comprising the hu6G4.2.5LV/L1N35X<sub>35</sub>. In still another embodiment, the  
 invention provides a humanized antibody or antibody fragment that comprises a light chain  
 variable domain comprising the hu6G4.2.5LV/L1N35A. In a further embodiment, the invention  
 20 provides a humanized antibody or antibody fragment that comprises a light chain variable  
 domain comprising the hu6G4.2.5LV/L1N35E.

The invention additionally provides a humanized antibody or antibody fragment that  
 comprises a light chain variable domain comprising the hu6G4.2.5LV/vL1-3X, and further  
 comprises a heavy chain variable domain comprising the hu6G4.2.5HV or hu6G4.2.5HV/vH1-  
 25 3Z. In another embodiment, the invention provides a humanized antibody or antibody fragment  
 that comprises a light chain variable domain comprising the hu6G4.2.5LV/vL1-3A, and further  
 comprises a heavy chain variable domain comprising the hu6G4.2.5HV or hu6G4.2.5HV/vH1-  
 3Z. In yet another embodiment, the invention provides a humanized antibody or antibody

fragment that comprises a light chain variable domain comprising the hu6G4.2.5LV/vL1-3A, and further comprises a heavy chain variable domain comprising the hu6G4.2.5HV/vH1-3A.

In a further embodiment, the invention provides a humanized antibody or antibody fragment that comprises a light chain variable domain comprising the hu6G4.2.5LV/L1N35X<sub>35</sub>, and further comprises a heavy chain variable domain comprising the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z. In another embodiment, the invention provides a humanized antibody or antibody fragment that comprises a light chain variable domain comprising the hu6G4.2.5LV/N35X<sub>35</sub>, and further comprises a heavy chain variable domain comprising the hu6G4.2.5HV/vH1-3A. In a preferred embodiment, the antibody or antibody fragment comprises a light chain variable domain comprising the hu6G4.2.5LV/L1N35X<sub>35</sub> and further comprises a humanized heavy chain comprising the amino acid sequence of 6G4.2.5HV11.

In an additional embodiment, the invention provides a humanized antibody or antibody fragment that comprises a light chain variable domain comprising the hu6G4.2.5LV/L1N35A, and further comprises a heavy chain variable domain comprising the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z. In another embodiment, the invention provides a humanized antibody or antibody fragment that comprises a light chain variable domain comprising the hu6G4.2.5LV/N35A, and further comprises a heavy chain variable domain comprising the hu6G4.2.5HV/vH1-3A. In still another embodiment, the humanized antibody or antibody fragment comprises a light chain variable domain comprising the hu6G4.2.5LV/L1N35A, and further comprises a heavy chain variable domain comprising the hu6G4.2.5HV. In a further embodiment, the humanized antibody or antibody fragment comprises a light chain variable domain comprising the hu6G4.2.5LV/L1N35E, and further comprises a heavy chain variable domain comprising the hu6G4.2.5HV. In a preferred embodiment, the antibody or antibody fragment comprises a light chain variable domain comprising the hu6G4.2.5LV/L1N35A and further comprises a humanized heavy chain comprising the amino acid sequence of 6G4.2.5HV11. In another preferred embodiment, the antibody or antibody fragment comprises a light chain variable domain comprising the hu6G4.2.5LV/L1N35E and further comprises a humanized heavy chain comprising the amino acid sequence of 6G4.2.5HV11.

The invention encompasses a single chain antibody fragment comprising the hu6G4.2.5LV/vL1-3X, with or without any additional amino acid sequence. In one embodiment, the invention provides a single chain antibody fragment comprising the hu6G4.2.5LV/vL1-3X without any associated heavy chain variable domain amino acid sequence, i.e. a single chain species that makes up one half of an Fv fragment. In another embodiment, the invention provides a single chain antibody fragment comprising the hu6G4.2.5LV/vL1-3A without any associated heavy chain variable domain amino acid sequence. In still another embodiment, the invention provides a single chain antibody fragment comprising the hu6G4.2.5LV/L1N35X<sub>35</sub> without any associated heavy chain variable domain amino acid sequence. In a preferred embodiment, the invention provides a single chain antibody fragment comprising the hu6G4.2.5LV/L1N35A without any associated heavy chain variable domain amino acid sequence. In another preferred embodiment, the invention provides a single chain antibody fragment comprising the hu6G4.2.5LV/L1N35E without any associated heavy chain variable domain amino acid sequence.

In one embodiment, the invention provides a single chain antibody fragment wherein the hu6G4.2.5LV/vL1-3X and the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment is a scFv species comprising the hu6G4.2.5LV/vL1-3X joined to the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z by means of a flexible peptide linker sequence, wherein the heavy chain and light chain variable domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fv species. In another embodiment, the single chain antibody fragment is a species comprising the hu6G4.2.5LV/vL1-3X joined to the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z by a linker that is too short to permit intramolecular pairing of the two variable domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In another embodiment, the invention provides a single chain antibody fragment wherein the hu6G4.2.5LV/vL1-3A and the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment

is a scFv species comprising the hu6G4.2.5LV/vL1-3A joined to the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z by means of a flexible peptide linker sequence, wherein the heavy chain and light chain variable domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fv species. In another embodiment, the single chain antibody fragment is a species comprising the hu6G4.2.5LV/vL1-3A joined to the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z by a linker that is too short to permit intramolecular pairing of the two variable domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In yet another embodiment, the invention provides a single chain antibody fragment wherein the hu6G4.2.5LV/vL1-3A and the hu6G4.2.5HV/vH1-3A are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment is a scFv species comprising the hu6G4.2.5LV/vL1-3A joined to the hu6G4.2.5HV/vH1-3A by means of a flexible peptide linker sequence, wherein the heavy chain and light chain variable domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fv species. In another embodiment, the single chain antibody fragment is a species comprising the hu6G4.2.5LV/vL1-3A joined to the hu6G4.2.5HV/vH1-3A by a linker that is too short to permit intramolecular pairing of the two variable domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In still another embodiment, the invention provides a single chain antibody fragment wherein the hu6G4.2.5LV/L1N35X<sub>35</sub> and the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment is a scFv species comprising the hu6G4.2.5LV/L1N35X<sub>35</sub> joined to the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z by means of a flexible peptide linker sequence, wherein the heavy chain and light chain variable domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fv species. In another embodiment, the single chain antibody fragment is a species comprising the hu6G4.2.5LV/L1N35X<sub>35</sub> joined to the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z by a linker that is too short to permit intramolecular pairing of the two variable domains, i.e. a single chain polypeptide monomer that forms a diabody upon

dimerization with another monomer.

In a further embodiment, the invention provides a single chain antibody fragment wherein the hu6G4.2.5LV/L1N35X<sub>35</sub> and the hu6G4.2.5HV/vH1-3A are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment is a scFv species comprising the hu6G4.2.5LV/L1N35X<sub>35</sub> joined to the hu6G4.2.5HV/vH1-3A by means of a flexible peptide linker sequence, wherein the heavy chain and light chain variable domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fv species. In another embodiment, the single chain antibody fragment is a species comprising the hu6G4.2.5LV/L1N35X<sub>35</sub> joined to the hu6G4.2.5HV/vH1-3A by a linker that is too short to permit intramolecular pairing of the two variable domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In an additional embodiment, the invention provides a single chain antibody fragment wherein the hu6G4.2.5LV/L1N35A and the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment is a scFv species comprising the hu6G4.2.5LV/L1N35A joined to the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z by means of a flexible peptide linker sequence, wherein the heavy chain and light chain variable domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fv species. In another embodiment, the single chain antibody fragment is a species comprising the hu6G4.2.5LV/L1N35A joined to the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z by a linker that is too short to permit intramolecular pairing of the two variable domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

Also provided herein is a single chain antibody fragment wherein the hu6G4.2.5LV/L1N35E and the hu6G4.2.5HV are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment is a scFv species comprising the hu6G4.2.5LV/L1N35E joined to the hu6G4.2.5HV by means of a flexible peptide linker sequence, wherein the heavy chain and light chain variable domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fv species. In another embodiment,

the single chain antibody fragment is a species comprising the hu6G4.2.5LV/L1N35E joined to the hu6G4.2.5HV by a linker that is too short to permit intramolecular pairing of the two variable domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

5 In still another embodiment, the invention provides a single chain antibody fragment wherein the hu6G4.2.5LV/L1N35A and the hu6G4.2.5HV/vH1-3A are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment is a scFv species comprising the hu6G4.2.5LV/L1N35A joined to the hu6G4.2.5HV/vH1-3A by means of a flexible peptide linker sequence, wherein the heavy chain and light chain variable  
10 domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fv species. In another embodiment, the single chain antibody fragment is a species comprising the hu6G4.2.5LV/L1N35A joined to the hu6G4.2.5HV/vH1-3A by a linker that is too short to permit intramolecular pairing of the two variable domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

15 In yet another embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/vL1-3X and a second polypeptide chain comprises the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds.

20 In still another embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/vL1-3X and a second polypeptide chain comprises the hu6G4.2.5HV/vH1-3A and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds. In a preferred embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/vL1-3X and a  
25 second polypeptide chain comprises the amino acid sequence of 6G4.2.5HV11 and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds.

In a further embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/vL1-

3A and a second polypeptide chain comprises the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds.

In still another embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/vL1-3A and a second polypeptide chain comprises the hu6G4.2.5HV/vH1-3A and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds. In a preferred embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/vL1-3A and a second polypeptide chain comprises the amino acid sequence of 6G4.2.5HV11 and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds.

The invention also encompasses an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/L1N35X<sub>35</sub> and a second polypeptide chain comprises the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds.

In still another embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/L1N35X<sub>35</sub> and a second polypeptide chain comprises the hu6G4.2.5HV/vH1-3A and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds. In a preferred embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/L1N35X<sub>35</sub> and a second polypeptide chain comprises the amino acid sequence of 6G4.2.5HV11 and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds.

The invention further encompasses an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/L1N35A and a second polypeptide chain comprises the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds.

The invention also encompasses an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/L1N35E and a

second polypeptide chain comprises the hu6G4.2.5HV and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds.

In still another embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/L1N35A and a second polypeptide chain comprises the hu6G4.2.5HV/vH1-3A and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds. In a preferred embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/L1N35A and a second polypeptide chain comprises the amino acid sequence of 6G4.2.5HV11 and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds. In another preferred embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5LV/L1N35E and a second polypeptide chain comprises the amino acid sequence of 6G4.2.5HV11 and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds.

In a preferred embodiment, any of the foregoing two-chain antibody fragments are selected from the group consisting of Fab, Fab', Fab'-SH, Fv, and F(ab')<sub>2</sub>. In another preferred embodiment, the antibody fragment is selected from the group consisting of Fab, Fab', Fab'-SH, Fv, and F(ab')<sub>2</sub>, wherein the antibody fragment comprises one polypeptide chain comprising the hu6G4.2.5LV/L1N35X<sub>35</sub> and a second polypeptide chain comprising the hu6G4.2.5HV. In yet another preferred embodiment, the antibody fragment is selected from the group consisting of Fab, Fab', Fab'-SH, Fv, and F(ab')<sub>2</sub>, wherein the antibody fragment comprises one polypeptide chain comprising the hu6G4.2.5LV/L1N35A and a second polypeptide chain comprising the hu6G4.2.5HV. In a further preferred embodiment, the antibody fragment is selected from the group consisting of Fab, Fab', Fab'-SH, Fv, and F(ab')<sub>2</sub>, wherein the antibody fragment comprises one polypeptide chain comprising the hu6G4.2.5LV/L1N35E and a second polypeptide chain comprising the hu6G4.2.5HV. In still another preferred embodiment, the antibody fragment is a F(ab')<sub>2</sub> that comprises one polypeptide chain comprising the hu6G4.2.5LV/L1N35A and a second polypeptide chain comprising the amino acid sequence of



6G4.2.5HV11. In an additional preferred embodiment, the antibody fragment is a F(ab')<sub>2</sub> that comprises one polypeptide chain comprising the hu6G4.2.5LV/L1N35E and a second polypeptide chain comprising the amino acid sequence of 6G4.2.5HV11.

The invention also provides an antibody or antibody fragment comprising a light chain variable domain containing the hu6G4.2.5LV/vL1-3X and optionally further comprising a heavy chain variable domain containing the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z, wherein the light chain variable domain, and optionally the heavy chain variable domain, is (are) fused to an additional moiety, such as a immunoglobulin constant domain. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

The invention additionally provides an antibody or antibody fragment comprising a light chain variable domain containing the hu6G4.2.5LV/vL1-3X and optionally further comprising a heavy chain variable domain containing the hu6G4.2.5HV/vH1-3A, wherein the light chain variable domain, and optionally the heavy chain variable domain, is (are) fused to an additional moiety, such as a immunoglobulin constant domain. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

The invention further provides an antibody or antibody fragment comprising a light chain variable domain containing the hu6G4.2.5LV/L1N35X<sub>35</sub> and optionally further comprising a heavy chain variable domain containing the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z, wherein the

light chain variable domain, and optionally the heavy chain variable domain, is (are) fused to an additional moiety, such as a immunoglobulin constant domain. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

The invention additionally provides an antibody or antibody fragment comprising a light chain variable domain containing the hu6G4.2.5LV/L1N35X<sub>35</sub> and optionally further comprising a heavy chain variable domain containing the hu6G4.2.5HV/vH1-3A, wherein the light chain variable domain, and optionally the heavy chain variable domain, is (are) fused to an additional moiety, such as a immunoglobulin constant domain. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

The invention also encompasses an antibody or antibody fragment comprising a light chain variable domain containing the hu6G4.2.5LV/L1N35A and optionally further comprising a heavy chain variable domain containing the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z, wherein the light chain variable domain, and optionally the heavy chain variable domain, is (are) fused to an additional moiety, such as a immunoglobulin constant domain. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant

domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

The invention additionally provides an antibody or antibody fragment comprising a light chain variable domain containing the hu6G4.2.5LV/L1N35A and optionally further comprising a heavy chain variable domain containing the hu6G4.2.5HV/vH1-3A, wherein the light chain variable domain, and optionally the heavy chain variable domain, is (are) fused to an additional moiety, such as a immunoglobulin constant domain. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

The invention additionally encompasses an antibody or antibody fragment comprising a light chain variable domain containing the hu6G4.2.5LV/L1N35A and optionally further comprising a heavy chain containing the amino acid sequence of 6G4.2.5HV11, wherein the light chain variable domain, and optionally the heavy chain, is (are) fused to an additional moiety, such as immunoglobulin constant domain sequences. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

The invention further encompasses an antibody or antibody fragment comprising a light chain variable domain containing the hu6G4.2.5LV/L1N35E and optionally further comprising a heavy chain containing the amino acid sequence of 6G4.2.5HV11, wherein the light chain variable domain, and optionally the heavy chain, is (are) fused to an additional moiety, such as

immunoglobulin constant domain sequences. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

In a preferred embodiment, the antibody or antibody fragment comprises a light chain variable domain containing the hu6G4.2.5LV/vL1-3X, and further comprises the hu6G4.2.5HV or hu6G4.2.5HV/vH1-3Z in a heavy chain that is fused to or contains a leucine zipper sequence. The leucine zipper can increase the affinity or production efficiency of the antibody or antibody fragment of interest. Suitable leucine zipper sequences include the jun and fos leucine zippers taught by Kostelney *et al.*, *J. Immunol.*, 148: 1547-1553 (1992) and the GCN4 leucine zipper described in the Examples below.

In particular, the invention provides an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that any amino acid other than Asn (denoted as "X<sub>35</sub>") is substituted for Asn at amino acid position 35 (herein referred to as "6G4.2.5LV11N35X<sub>35</sub>").

In another embodiment, the invention provides an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that any amino acid other than Ser (denoted as "X<sub>26</sub>") is substituted for Ser at amino acid position 26 (herein referred to as "6G4.2.5LV11S26X<sub>26</sub>").

In yet another embodiment, the invention provides an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that any amino acid other than His (denoted as "X<sub>98</sub>") is substituted

for His at amino acid position 98 (herein referred to as "6G4.2.5LV11H98X<sub>98</sub>").

In still another embodiment, the invention provides an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that any amino acid other than Ser (denoted as "X<sub>26</sub>") is substituted for Ser at amino acid position 26 and any amino acid other than Asn (denoted as "X<sub>35</sub>") is substituted for Asn at amino acid position 35 (herein referred to as "6G4.2.5LV11S26X<sub>26</sub>/N35X<sub>35</sub>").

In a further embodiment, the invention provides an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that any amino acid other than Asn (denoted as "X<sub>35</sub>") is substituted for Asn at amino acid position 35 and any amino acid other than His (denoted as "X<sub>98</sub>") is substituted for His at amino acid position 98 (herein referred to as "6G4.2.5LV11N35X<sub>35</sub>/H98X<sub>98</sub>").

In an additional embodiment, the invention provides an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that any amino acid other than Ser (denoted as "X<sub>26</sub>") is substituted for Ser at amino acid position 26 and any amino acid other than His (denoted as "X<sub>98</sub>") is substituted for His at amino acid position 98 (herein referred to as "6G4.2.5LV11S26X<sub>26</sub>/H98X<sub>98</sub>").

The invention also encompasses an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that any amino acid other than Ser (denoted as "X<sub>26</sub>") is substituted for Ser at amino acid position 26, any amino acid other than Asn (denoted as "X<sub>35</sub>") is substituted for Asn at amino acid position 35 and any amino acid other than His (denoted as "X<sub>98</sub>") is substituted for

His at amino acid position 98 (herein referred to as "6G4.2.5LV11S26X<sub>26</sub>/N35X<sub>35</sub>/H98X<sub>98</sub>").

Additionally, the invention provides an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence (SEQ ID NO: 56) of Fig. 36 (herein referred to as "6G4.2.5LV11N35A").

Further provided herein is an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence (SEQ ID NO: 62) of Fig. 45 (herein referred to as "6G4.2.5LV11N35E").

In another embodiment, the invention provides an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that Ala is substituted for Ser at amino acid position 26 (herein referred to as "6G4.2.5LV11S26A").

In yet another embodiment, the invention provides an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that Ala is substituted for His at amino acid position 98 (herein referred to as "6G4.2.5LV11H98A").

In still another embodiment, the invention provides an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that Ala is substituted for Ser at amino acid position 26 and Ala is substituted for Asn at amino acid position 35 (herein referred to as "6G4.2.5LV11S26A/N35A").

In a further embodiment, the invention provides an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that Ala is substituted for Ser at amino acid position 26 and Ala is

substituted for His at amino acid position 98 (herein referred to as "6G4.2.5LV11S26A/H98A").

The invention also encompasses an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that Ala is substituted for Asn at amino acid position 35 and Ala is substituted for His at amino acid position 98 (herein referred to as "6G4.2.5LV11N35A/H98A").

The invention further encompasses an antibody or antibody fragment comprising a light chain comprising the amino acid sequence of amino acids 1-219 of the variant humanized anti-IL-8 6G4.2.5v11 light chain polypeptide amino acid sequence of Fig. 31B (SEQ ID NO: 51) with the proviso that Ala is substituted for Ser at amino acid position 26, Ala is substituted for Asn at amino acid position 35, and Ala is substituted for His at amino acid position 98 (herein referred to as "6G4.2.5LV11S26A/N35A/H98A").

The invention provides a single chain antibody fragment comprising a variant light chain selected from the group consisting of 6G4.2.5LV11N35X<sub>35</sub>, 6G4.2.5LV11S26X<sub>26</sub>, 6G4.2.5LV11H98X<sub>98</sub>, 6G4.2.5LV11S26X<sub>26</sub>/N35X<sub>35</sub>, 6G4.2.5LV11N35X<sub>35</sub>/H98X<sub>98</sub>, 6G4.2.5LV11S26X<sub>26</sub>/H98X<sub>98</sub>, and 6G4.2.5LV11S26X<sub>26</sub>/N35X<sub>35</sub>/H98X<sub>98</sub>, with or without any additional amino acid sequence. It will be understood that the group consisting of 6G4.2.5LV11N35X<sub>35</sub>, 6G4.2.5LV11S26X<sub>26</sub>, 6G4.2.5LV11H98X<sub>98</sub>, 6G4.2.5LV11S26X<sub>26</sub>/N35X<sub>35</sub>, 6G4.2.5LV11N35X<sub>35</sub>/H98X<sub>98</sub>, 6G4.2.5LV11S26X<sub>26</sub>/H98X<sub>98</sub>, and 6G4.2.5LV11S26X<sub>26</sub>/N35X<sub>35</sub>/H98X<sub>98</sub>, is collectively referred to herein as the "group of 6G4.2.5LV11X variants", and that individual members of this group are generically referred to herein as a "6G4.2.5LV11X variant." In one embodiment, the invention provides a single chain antibody fragment comprising a 6G4.2.5LV11X variant without any associated heavy chain amino acid sequence, i.e. a single chain species that makes up one half of a Fab fragment. In a preferred embodiment, the invention provides a 6G4.2.5LV11N35X<sub>35</sub> variant without any associated heavy chain amino acid sequence.

The invention encompasses a single chain antibody fragment comprising a variant light chain selected from the group consisting of 6G4.2.5LV11N35A, 6G4.2.5LV11S26A,

6G4.2.5LV11H98A, 6G4.2.5LV11S26A/ N35A, 6G4.2.5LV11N35A/ H98A,  
 6G4.2.5LV11S26A/H98A, and 6G4.2.5LV11S26A/ N35A/H98A, with or without any  
 additional amino acid sequence. It will be understood that the group consisting of  
 6G4.2.5LV11N35A, 6G4.2.5LV11S26A, 6G4.2.5LV11H98A, 6G4.2.5LV11S26A/ N35A,  
 5 6G4.2.5LV11N35A/ H98A, 6G4.2.5LV11S26A/H98A, and 6G4.2.5LV11S26A/ N35A/H98A is  
 collectively referred to herein as the "group of 6G4.2.5LV11A variants", and that individual  
 members of this group are generically referred to herein as a "6G4.2.5LV11A variant." In one  
 embodiment, the invention provides a single chain antibody fragment comprising a  
 6G4.2.5LV11A variant without any associated heavy chain amino acid sequence, i.e. a single  
 10 chain species that makes up one half of a Fab fragment. In a preferred embodiment, the  
 invention provides the 6G4.2.5LV11N35A without any associated heavy chain amino acid  
 sequence.

Further provided herein are an antibody or antibody fragment comprising a light chain  
 comprising a 6G4.2.5LV11X variant, and further comprising a heavy chain comprising the  
 15 6G4.2.5HV11. In a preferred embodiment, the invention provides an antibody or antibody  
 fragment comprising a 6G4.2.5LV11N35X<sub>35</sub> variant and further comprising the 6G4.2.5HV11.  
 In a preferred embodiment, the invention provides an antibody or antibody fragment comprising  
 the 6G4.2.5LV11N35A and further comprising the 6G4.2.5HV11. In another preferred  
 embodiment, the invention provides an antibody or antibody fragment comprising the  
 20 6G4.2.5LV11N35E and further comprising the 6G4.2.5HV11.

In one embodiment, the invention provides a single chain antibody fragment wherein a  
 6G4.2.5LV11X variant and the 6G4.2.5HV11 are contained in a single chain polypeptide  
 species. In a preferred embodiment, the single chain antibody fragment comprises a  
 6G4.2.5LV11X variant joined to the 6G4.2.5HV11 by means of a flexible peptide linker  
 25 sequence, wherein the heavy chain and light chain domains can associate in a "dimeric" structure  
 analogous to that formed in a two-chain Fab species. In another embodiment, the single chain  
 antibody fragment is a species comprising a 6G4.2.5LV11X variant joined to the 6G4.2.5HV11  
 by a linker that is too short to permit intramolecular pairing of complementary domains, i.e. a



single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In still another embodiment, the invention provides a single chain antibody fragment wherein a 6G4.2.5LV11N35X<sub>35</sub> variant and the 6G4.2.5HV11 are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment comprises a 6G4.2.5LV11N35X<sub>35</sub> variant joined to the 6G4.2.5HV11 by means of a flexible peptide linker sequence, wherein the heavy chain and light chain domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fab species. In another embodiment, the single chain antibody fragment is a species comprising a 6G4.2.5LV11N35X<sub>35</sub> variant joined to the 6G4.2.5HV11 by a linker that is too short to permit intramolecular pairing of complementary domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In a further embodiment, the invention provides a single chain antibody fragment wherein the 6G4.2.5LV11N35A and the 6G4.2.5HV11 are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment comprises the 6G4.2.5LV11N35A joined to the 6G4.2.5HV11 by means of a flexible peptide linker sequence, wherein the heavy chain and light chain domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fab species. In another embodiment, the single chain antibody fragment is a species comprising the 6G4.2.5LV11N35A joined to the 6G4.2.5HV11 by a linker that is too short to permit intramolecular pairing of complementary domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In an additional embodiment, the invention provides a single chain antibody fragment wherein the 6G4.2.5LV11N35E and the 6G4.2.5HV11 are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment comprises the 6G4.2.5LV11N35E joined to the 6G4.2.5HV11 by means of a flexible peptide linker sequence, wherein the heavy chain and light chain domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fab species. In another embodiment, the single chain antibody fragment is a species comprising the 6G4.2.5LV11N35E joined to the 6G4.2.5HV11 by

a linker that is too short to permit intramolecular pairing of complementary domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In yet another embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises a 6G4.2.5LV11X variant and a second polypeptide chain comprises the 6G4.2.5HV11 and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds. In still another embodiment, the invention provides an antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises a 6G4.2.5LV11N35X<sub>35</sub> variant and a second polypeptide chain comprises the 6G4.2.5HV11 and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds. In a preferred embodiment, any of the foregoing two-chain antibody fragments is selected from the group consisting of Fab, Fab', Fab'-SH, and F(ab')<sub>2</sub>. In still another preferred embodiment, the two-chain antibody fragment is a F(ab')<sub>2</sub> wherein one polypeptide chain comprises the 6G4.2.5LV11N35A and the second polypeptide chain comprises the 6G4.2.5HV11. In a further preferred embodiment, the antibody fragment is a Fab, Fab', Fab'-SH, or F(ab')<sub>2</sub> wherein one polypeptide chain comprises the 6G4.2.5LV11N35E and the second polypeptide chain comprises the 6G4.2.5HV11. A particularly preferred embodiment, the antibody fragment is the 6G4V11N35A F(ab')<sub>2</sub> GCN4 leucine zipper species described in the Examples below. In another particularly preferred embodiment, the antibody fragment is the 6G4V11N35E F(ab')<sub>2</sub> GCN4 leucine zipper species described in the Examples below. In yet another particularly preferred embodiment, the antibody fragment is the 6G4V11N35E Fab described in the Examples below.

The invention also provides an antibody or antibody fragment comprising a light chain containing a 6G4.2.5LV11X variant and optionally further comprising a heavy chain containing the 6G4.2.5HV11, wherein the light chain, and optionally the heavy chain, is (are) fused to an additional moiety, such as additional immunoglobulin constant domain sequence. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant

regions, and that such constant regions can be obtained from any human or animal species.

Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

5 The invention additionally provides an antibody or antibody fragment comprising a light chain containing a 6G4.2.5LV11N35X<sub>35</sub> variant and optionally further comprising a heavy chain containing the 6G4.2.5HV11, wherein the light chain, and optionally the heavy chain, is (are) fused to an additional moiety, such as additional immunoglobulin constant domain sequence. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that  
10 constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

15 The invention further provides an antibody or antibody fragment comprising a light chain containing the 6G4.2.5LV11N35A and optionally further comprising a heavy chain containing the 6G4.2.5HV11, wherein the light chain, and optionally the heavy chain, is (are) fused to an additional moiety, such as additional immunoglobulin constant domain sequence. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions  
20 of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

25 The invention further provides an antibody or antibody fragment comprising a light chain containing the 6G4.2.5LV11N35E and optionally further comprising a heavy chain containing the 6G4.2.5HV11, wherein the light chain, and optionally the heavy chain, is (are) fused to an additional moiety, such as additional immunoglobulin constant domain sequence. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species

with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species.

Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

In a preferred embodiment, the antibody or antibody fragment comprises a light chain containing a 6G4.2.5LV11X variant, and further comprises the 6G4.2.5HV11 in a heavy chain that is fused to or contains a leucine zipper sequence. The leucine zipper can increase the affinity or production efficiency of the antibody or antibody fragment of interest. Suitable leucine zipper sequences include the jun and fos leucine zippers taught by Kostelney *et al.*, *J. Immunol.*, 148: 1547-1553 (1992) and the GCN4 leucine zipper described in the Examples below. In another preferred embodiment, the antibody or antibody fragment comprises a light chain containing the 6G4.2.5LV11N35A, and further comprises a heavy chain containing the 6G4.2.5HV11 fused to the GCN4 leucine zipper. In yet another preferred embodiment, the antibody or antibody fragment comprises a light chain containing the 6G4.2.5LV11N35E, and further comprises a heavy chain containing the 6G4.2.5HV11 fused to the GCN4 leucine zipper.

#### B. 6G4.2.5HV VARIANTS

The invention provides humanized antibodies and antibody fragments comprising the CDRs of a 6G4.2.5HV CDR variant. The use of a 6G4.2.5HV CDRs variant in the humanized antibodies and antibody fragments of the invention confer the advantages of higher affinity for human IL-8 and/or improved recombinant manufacturing economy.

A heavy chain variable domain comprising the CDRs of a 6G4.2.5HV CDRs variant can be humanized in conjunction with a light chain comprising the CDRs of 6G4.2.5LV or the CDRs of a 6G4.2.5LV CDRs variant, essentially as described in Section (II)(2)(A) above. In one embodiment, the invention provides a humanized antibody or antibody fragment comprising a 6G4.2.5HV CDRs variant selected from the group consisting of 6G4.2.5HV/H1S31Z<sub>31</sub>, 6G4.2.5HV/H2S54Z<sub>54</sub>, and 6G4.2.5HV/H1S31Z<sub>31</sub>/H2S54Z<sub>54</sub>. In addition, the invention provides

a humanized antibody or antibody fragment comprising a 6G4.2.5HV CDRs variant selected from the group consisting of 6G4.2.5HV/H1S31A, 6G4.2.5HV/H2S54A, and 6G4.2.5HV/H1S31A/H2S54A. In particular, the 6G4.2.5HV CDRs variants can be used to construct a humanized antibody or antibody comprising the hu6G4.2.5HV/vH1-3Z as described in Section (II)(2)(A) above.

The invention additionally provides a humanized antibody or antibody fragment that comprises a heavy chain variable domain comprising the hu6G4.2.5HV/vH1-3Z, and further comprises a light chain variable domain comprising the hu6G4.2.5LV or hu6G4.2.5LV/vL1-3X.

The invention further encompasses a single chain humanized antibody fragment comprising the hu6G4.2.5HV/vH1-3Z, with or without any additional amino acid sequence. In one embodiment, the invention provides a single chain antibody fragment comprising the hu6G4.2.5HV/vH1-3Z without any associated heavy chain variable domain amino acid sequence, i.e. a single chain species that makes up one half of an Fv fragment.

In one embodiment, the invention provides a single chain humanized antibody fragment wherein the hu6G4.2.5HV/vH1-3Z and the hu6G4.2.5LV or hu6G4.2.5LV/vL1-3X are contained in a single chain polypeptide species. In a preferred embodiment, the single chain antibody fragment is a scFv species comprising the hu6G4.2.5HV/vH1-3Z joined to the hu6G4.2.5LV or hu6G4.2.5LV/vL1-3X by means of a flexible peptide linker sequence, wherein the heavy chain and light chain variable domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fv species. In another embodiment, the single chain antibody fragment is a species comprising the hu6G4.2.5HV/vH1-3Z joined to the hu6G4.2.5LV or hu6G4.2.5LV/vL1-3X by a linker that is too short to permit intramolecular pairing of the two variable domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In yet another embodiment, the invention provides a humanized antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises the hu6G4.2.5HV/vH1-3Z and a second polypeptide chain comprises the hu6G4.2.5LV or hu6G4.2.5LV/vL1-3X and the two polypeptide chains are covalently linked by one or more

interchain disulfide bonds. In a preferred embodiment, the foregoing two-chain antibody fragment is selected from the group consisting of Fab, Fab', Fab'-SH, Fv, and F(ab')<sub>2</sub>.

The invention also provides a humanized antibody or antibody fragment comprising a heavy chain variable domain containing the hu6G4.2.5HV/vH1-3Z and optionally further comprising a light chain variable domain containing the hu6G4.2.5LV or hu6G4.2.5LV/vL1-3X, wherein the heavy chain variable domain, and optionally the light chain variable domain, is (are) fused to an additional moiety, such as an immunoglobulin constant domain. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.*

In a preferred embodiment, the humanized antibody or antibody fragment comprises the hu6G4.2.5HV/vH1-3Z in a heavy chain that is fused to or contains a leucine zipper sequence. The leucine zipper can increase the affinity or production efficiency of the antibody or antibody fragment of interest. Suitable leucine zipper sequences include the jun and fos leucine zippers taught by Kostelney *et al.*, *J. Immunol.*, 148: 1547-1553 (1992) and the GCN4 leucine zipper described in the Examples below.

In addition, the invention provides a humanized antibody or antibody fragment comprising a heavy chain comprising the amino acid sequence of amino acids 1-230 of the 6G4.2.5HV11 polypeptide amino acid sequence of Figs. 37A-37B (SEQ ID NO: 60) with the proviso that Ala is substituted for Ser at amino acid position 31 (hereinafter referred to as "6G4.2.5HV11S31A").

In another embodiment, the invention provides a humanized antibody or antibody fragment comprising a heavy chain comprising the amino acid sequence of amino acids 1-230 of the 6G4.2.5HV11 polypeptide amino acid sequence of Figs. 37A-37B (SEQ ID NO: 60) with the proviso that Ala is substituted for Ser at amino acid position 54 (hereinafter referred to as

"6G4.2.5HV11S54A").

In yet another embodiment, the invention provides a humanized antibody or antibody fragment comprising a heavy chain comprising the amino acid sequence of amino acids 1-230 of the 6G4.2.5HV11 polypeptide amino acid sequence of Figs. 37A-37B (SEQ ID NO: 60) with the proviso that Ala is substituted for Ser at amino acid position 31 and Ala is substituted for Ser at amino acid position 54 (hereinafter referred to as "6G4.2.5HV11S31A/S54A").

Further provided herein is a humanized antibody or antibody fragment that comprises any of the light and heavy chain combinations listed in Tables 1-2 below.

Table 1

Heavy Chain	Light Chain
6G4.2.5HV11S31A	6G4.2.5LV11
6G4.2.5HV11S31A	6G4.2.5LV11N35A
6G4.2.5HV11S31A	6G4.2.5LV11S26A
6G4.2.5HV11S31A	6G4.2.5LV11H98A
6G4.2.5HV11S31A	6G4.2.5LV11S26A/N35A
6G4.2.5HV11S31A	6G4.2.5LV11S26A/H98A
6G4.2.5HV11S31A	6G4.2.5LV11N35A/H98A
6G4.2.5HV11S31A	6G4.2.5LV11S26A/N35A/H98A
6G4.2.5HV11S54A	6G4.2.5LV11
6G4.2.5HV11S54A	6G4.2.5LV11N35A
6G4.2.5HV11S54A	6G4.2.5LV11S26A
6G4.2.5HV11S54A	6G4.2.5LV11H98A
6G4.2.5HV11S54A	6G4.2.5LV11S26A/N35A
6G4.2.5HV11S54A	6G4.2.5LV11S26A/H98A
6G4.2.5HV11S54A	6G4.2.5LV11N35A/H98A
6G4.2.5HV11S54A	6G4.2.5LV11S26A/N35A/H98A
6G4.2.5HV11S31A/S54A	6G4.2.5LV11
6G4.2.5HV11S31A/S54A	6G4.2.5LV11N35A
6G4.2.5HV11S31A/S54A	6G4.2.5LV11S26A
6G4.2.5HV11S31A/S54A	6G4.2.5LV11H98A
6G4.2.5HV11S31A/S54A	6G4.2.5LV11S26A/N35A
6G4.2.5HV11S31A/S54A	6G4.2.5LV11S26A/H98A
6G4.2.5HV11S31A/S54A	6G4.2.5LV11N35A/H98A
6G4.2.5HV11S31A/S54A	6G4.2.5LV11S26A/N35A/H98A

Table 2

	Heavy Chain	Light Chain
5	6G4.2.5HV11S31A	6G4.2.5LV11
	6G4.2.5HV11S31A	6G4.2.5LV11N35X <sub>35</sub>
	6G4.2.5HV11S31A	6G4.2.5LV11S26X <sub>26</sub>
	6G4.2.5HV11S31A	6G4.2.5LV11H98X <sub>98</sub>
	6G4.2.5HV11S31A	6G4.2.5LV11S26X <sub>26</sub> /N35X <sub>35</sub>
10	6G4.2.5HV11S31A	6G4.2.5LV11S26X <sub>26</sub> /H98X <sub>98</sub>
	6G4.2.5HV11S31A	6G4.2.5LV11N35X <sub>35</sub> /H98X <sub>98</sub>
	6G4.2.5HV11S31A	6G4.2.5LV11S26X <sub>26</sub> /N35X <sub>35</sub> /H98X <sub>98</sub>
	6G4.2.5HV11S54A	6G4.2.5LV11
	6G4.2.5HV11S54A	6G4.2.5LV11N35X <sub>35</sub>
15	6G4.2.5HV11S54A	6G4.2.5LV11S26X <sub>26</sub>
	6G4.2.5HV11S54A	6G4.2.5LV11H98X <sub>98</sub>
	6G4.2.5HV11S54A	6G4.2.5LV11S26X <sub>26</sub> /N35X <sub>35</sub>
	6G4.2.5HV11S54A	6G4.2.5LV11S26X <sub>26</sub> /H98X <sub>98</sub>
	6G4.2.5HV11S54A	6G4.2.5LV11N35X <sub>35</sub> /H98X <sub>98</sub>
20	6G4.2.5HV11S54A	6G4.2.5LV11S26X <sub>26</sub> /N35X <sub>35</sub> /H98X <sub>98</sub>
	6G4.2.5HV11S31A/S54A	6G4.2.5LV11
	6G4.2.5HV11S31A/S54A	6G4.2.5LV11N35X <sub>35</sub>
	6G4.2.5HV11S31A/S54A	6G4.2.5LV11S26X <sub>26</sub>
	6G4.2.5HV11S31A/S54A	6G4.2.5LV11H98X <sub>98</sub>
25	6G4.2.5HV11S31A/S54A	6G4.2.5LV11S26X <sub>26</sub> /N35X <sub>35</sub>
	6G4.2.5HV11S31A/S54A	6G4.2.5LV11S26X <sub>26</sub> /H98X <sub>98</sub>
	6G4.2.5HV11S31A/S54A	6G4.2.5LV11N35X <sub>35</sub> /H98X <sub>98</sub>
	6G4.2.5HV11S31A/S54A	6G4.2.5LV11S26X <sub>26</sub> /N35X <sub>35</sub> /H98X <sub>98</sub>
30		

The invention encompasses a single chain humanized antibody fragment comprising a variant heavy chain selected from the group consisting of 6G4.2.5HV11S31A, 6G4.2.5HV11S54A, and 6G4.2.5HV11S31A/ S54A, with or without any additional amino acid sequence. It will be understood that the group consisting of 6G4.2.5HV11S31A, 6G4.2.5HV11S54A, and 6G4.2.5HV11S31A/ S54A is collectively referred to herein as the "group of 6G4.2.5HV11A variants", and that individual members of this group are generically referred to herein as a "6G4.2.5HV11A variant." In one embodiment, the invention provides a



single chain humanized antibody fragment comprising a 6G4.2.5HV11A variant without any associated light chain amino acid sequence, i.e. a single chain species that makes up one half of a Fab fragment.

Further provided herein are a humanized antibody or antibody fragment comprising a heavy chain comprising a 6G4.2.5HV11A variant, and further comprising a light chain comprising a 6G4.2.5LV11A variant or a 6G4.2.5LV11X variant. In another embodiment, the humanized antibody or antibody fragment comprises any combination of light and heavy chains listed in Tables 1 and 2 above. In one embodiment, the invention provides a humanized antibody or antibody fragment comprising a 6G4.2.5HV11A variant and further comprising the 6G4.2.5LV11N35X<sub>35</sub>. In a preferred embodiment, the invention provides a humanized antibody or antibody fragment comprising a 6G4.2.5HV11A variant and further comprising the 6G4.2.5LV11N35A.

In yet another embodiment, the invention provides a single chain humanized antibody fragment wherein a 6G4.2.5HV11A variant and the 6G4.2.5LV11 are contained in a single chain polypeptide species. In another embodiment, the invention provides a single chain humanized antibody fragment wherein any pair of light and heavy chains listed in Tables 1-2 above is contained in a single chain polypeptide species. In yet another embodiment, the invention provides a single chain humanized antibody fragment wherein a 6G4.2.5HV11A variant and a 6G4.2.5LV11X variant are contained in a single chain polypeptide species. In still another embodiment, the invention provides a single chain humanized antibody fragment wherein a 6G4.2.5HV11A variant and a 6G4.2.5LV11N35X<sub>35</sub> variant are contained in a single chain polypeptide species. In an additional embodiment, the invention provides a single chain humanized antibody fragment wherein a 6G4.2.5HV11A variant and the 6G4.2.5LV11N35A variant are contained in a single chain polypeptide species.

In a preferred embodiment, the single chain humanized antibody fragment comprises a 6G4.2.5HV11A variant joined to a 6G4.2.5LV11X variant, 6G4.2.5LV11N35X<sub>35</sub> variant, 6G4.2.5LV11N35A variant, or 6G4.2.5LV11 by means of a flexible peptide linker sequence, wherein the heavy chain and light chain domains can associate in a "dimeric" structure analogous

to that formed in a two-chain Fab species. In a further embodiment, the single chain humanized antibody fragment is a species comprising a 6G4.2.5HV11A variant joined to a 6G4.2.5LV11X variant, 6G4.2.5LV11N35X<sub>35</sub> variant, 6G4.2.5LV11N35A variant, or 6G4.2.5LV11 by a linker that is too short to permit intramolecular pairing of complementary domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In still another embodiment, the single chain humanized antibody fragment comprises any pair of light and heavy chains listed in Table 1 above joined by means of a flexible peptide linker sequence, wherein the heavy chain and light chain domains can associate in a "dimeric" structure analogous to that formed in a two-chain Fab species. In an additional embodiment, the single chain humanized antibody fragment comprises any pair of light and heavy chains listed in Tables 1-2 above joined by a linker that is too short to permit intramolecular pairing of complementary domains, i.e. a single chain polypeptide monomer that forms a diabody upon dimerization with another monomer.

In yet another embodiment, the invention provides a humanized antibody fragment comprising a plurality of polypeptide chains, wherein one polypeptide chain comprises a 6G4.2.5HV11A variant and a second polypeptide chain comprises a 6G4.2.5LV11X variant, 6G4.2.5LV11N35X<sub>35</sub> variant, 6G4.2.5LV11N35A variant, or 6G4.2.5LV11, and the two polypeptide chains are covalently linked by one or more interchain disulfide bonds. In a preferred embodiment, the foregoing two-chain antibody fragment is selected from the group consisting of Fab, Fab', Fab'-SH, and F(ab')<sub>2</sub>.

In an additional embodiment, the invention provides a two-chain humanized antibody fragment comprising any pair of heavy and light chains listed in Tables 1-2 above, wherein each chain is contained on a separate molecule. In another embodiment, the two-chain antibody fragment comprising any pair of heavy and light chains listed in Tables 1-2 above is selected from the group consisting of Fab, Fab', Fab'-SH, and F(ab')<sub>2</sub>. In a preferred embodiment, the two-chain humanized antibody fragment is a F(ab')<sub>2</sub> comprising any pair of heavy and light chains listed in Tables 1-2 above. In another preferred embodiment, the two-chain humanized antibody fragment is a F(ab')<sub>2</sub> wherein one polypeptide chain comprises a 6G4.2.5HV11A

variant and the second polypeptide chain comprises the 6G4.2.5LV11N35A.

The invention also provides a humanized antibody or antibody fragment comprising a heavy chain containing a 6G4.2.5HV11A variant and optionally further comprising a light chain containing a 6G4.2.5LV11X variant, 6G4.2.5LV11N35X<sub>35</sub> variant, 6G4.2.5LV11N35A, or 6G4.2.5HV11, wherein the heavy chain, and optionally the light chain, is (are) fused to an additional moiety, such as additional immunoglobulin constant domain sequence. Constant domain sequence can be added to the heavy chain and/or light chain sequence(s) to form species with full or partial length heavy and/or light chain(s). It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. Preferably, the constant domain sequence is human in origin. Suitable human constant domain sequences can be obtained from Kabat *et al.* (supra).

In a preferred embodiment, the humanized antibody or antibody fragment comprises a 6G4.2.5HV11A variant in a heavy chain that is fused to or contains a leucine zipper sequence. The leucine zipper can increase the affinity or production efficiency of the antibody or antibody fragment of interest. Suitable leucine zipper sequences include the jun and fos leucine zippers taught by Kostelney *et al.*, *J. Immunol.*, 148: 1547-1553 (1992) and the GCN4 leucine zipper described in the Examples below.

### C. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for IL-8, the other one is for any other antigen. For example, bispecific antibodies specifically binding a IL-8 and neurotrophic factor, or two different types of IL-8 polypeptides are within the scope of the present invention.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different

specificities (Milstein and Cuello, Nature 305:537 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829 published 13 May 1993, and in Traunecker *et al.*, EMBO J. 10:3655 (1991).

According to a different and more preferred approach, antibody-variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant-domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1), containing the site necessary for light-chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the maximum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the production of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. This asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. For further details of generating bispecific antibodies, see, for example, Suresh *et al.*, Methods in Enzymology 121:210 (1986).

According to another approach, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C<sub>H</sub>3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/00373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, *Science*, **229**: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')<sub>2</sub> fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby *et al.*, *J. Exp. Med.*,

175: 217-225 (1992) describe the production of a fully humanized bispecific antibody F(ab')<sub>2</sub> molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.*, **148(5)**:1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, **90**:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the VH and VL domains of one fragment are forced to pair with the complementary VL and VH domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.*, **152**:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al.* *J. Immunol.* **147**: 60 (1991).

#### 4. Production of Humanized Anti-IL-8 6G4.2.5 Monoclonal Antibody, Antibody Fragments, and Variants

The antibodies and antibody fragments of the invention can be produced using any convenient antibody manufacturing process known in the art. Typically, the antibody or antibody fragment is made using recombinant expression systems. A multiple polypeptide chain

antibody or antibody fragment species can be made in a single host cell expression system wherein the host cell produces each chain of the antibody or antibody fragment and assembles the polypeptide chains into a multimeric structure to form the antibody or antibody fragment in vivo, followed by recovery of the antibody or antibody fragment from the host cell. For example, suitable recombinant expression systems for the production of complete antibody or antibody fragment are described in Lucas *et al.*, Nucleic Acids Res., 24: 1774-1779 (1996). Alternatively, the separate polypeptide chains of the desired antibody or antibody fragment can be made in separate expression host cells, separately recovered from the respective host cells, and then mixed in vitro under conditions permitting the formation of the multi-subunit antibody or antibody fragment of interest. For example, U.S. Pat. No. 4,816,567 to Cabilly *et al.* and Carter *et al.*, Bio/Technology, 10: 163-167 (1992) provide methods for recombinant production of antibody heavy and light chains in separate expression hosts followed by assembly of antibody from separate heavy and light chains in vitro.

The following discussion of recombinant expression methods applies equally to the production of single chain antibody polypeptide species and multi-subunit antibody and antibody fragment species. All recombinant procedures for the production of antibody or antibody fragment provided below shall be understood to describe: (1) manufacture of single chain antibody species as the desired end-product; (2) manufacture of multi-subunit antibody or antibody fragment species by production of all subunits in a single host cell, subunit assembly in the host cell, optionally followed by host cell secretion of the multi-subunit end-product into the culture medium, and recovery of the multi-subunit end-product from the host cell and/or culture medium; and (3) manufacture of multi-subunit antibody or antibody fragment by production of subunits in separate host cells (optionally followed by host cell secretion of subunits into the culture medium), recovery of subunits from the respective host cells and/or culture media, followed by in vitro subunit assembly to form the multi-subunit end-product. In the case of a multi-subunit antibody or antibody fragment produced in a single host cell, it will be appreciated that production of the various subunits can be effected by expression of multiple polypeptide-encoding nucleic acid sequences carried on a single vector or by expression of polypeptide-

encoding nucleic acid sequences carried on multiple vectors contained in the host cell.

A. Construction of DNA Encoding Humanized 6G4.2.5 Monoclonal Antibodies, Antibody Fragments, and Variants

5           Following the selection of the humanized antibody or antibody fragment of the invention according to the methods described above, the practitioner can use the genetic code to design DNAs encoding the desired antibody or antibody fragment. In one embodiment, codons preferred by the expression host cell are used in the design of a DNA encoding the antibody or antibody fragment of interest. DNA encoding the desired antibody or antibody fragment can be  
10       prepared by a variety of methods known in the art. These methods include, but are not limited to, chemical synthesis by any of the methods described in Engels *et al.*, Agnew. Chem. Int. Ed. Engl., 28: 716-734 (1989), the entire disclosure of which is incorporated herein by reference, such as the triester, phosphite, phosphoramidite and H-phosphonate methods.

          A variation on the above procedures contemplates the use of gene fusions, wherein the  
15       gene(s) encoding the antibody or antibody fragment is associated, in the vector, with a gene encoding another protein or a fragment of another protein. This results in the antibody or antibody fragment being produced by the host cell as a fusion with another protein. The "other" protein is often a protein or peptide which can be secreted by the cell, making it possible to isolate and purify the desired protein from the culture medium and eliminating the necessity of  
20       destroying the host cells which arises when the desired protein remains inside the cell. Alternatively, the fusion protein can be expressed intracellularly. It is advantageous to use fusion proteins that are highly expressed.

          The use of gene fusions, though not essential, can facilitate the expression of heterologous proteins in *E. coli* as well as the subsequent purification of those gene products  
25       (Harris, T. J. R. in *Genetic Engineering*, Williamson, R., Ed., Academic, London, Vol. 4, p. 127(1983); Uhlen, M. & Moks, T., *Methods Enzymol.* 185:129-143 (1990)). Protein A fusions are often used because the binding of protein A, or more specifically the Z domain of protein A, to IgG provides an "affinity handle" for the purification of the fused protein (Nilsson, B. &



Abrahmsen, L. *Methods Enzymol.* **185**:144-161 (1990)). It has also been shown that many heterologous proteins are degraded when expressed directly in *E. coli*, but are stable when expressed as fusion proteins (Marston, F. A. O., *Biochem J.* **240**: 1 (1986)).

Fusion proteins can be cleaved using chemicals, such as cyanogen bromide, which cleaves at a methionine, or hydroxylamine, which cleaves between an Asn and Gly. Using standard recombinant DNA methodology, the nucleotide base pairs encoding these amino acids may be inserted just prior to the 5' end of the antibody or antibody fragment gene(s).

Alternatively, one can employ proteolytic cleavage of fusion proteins, which has been recently reviewed (Carter, P. (1990) in *Protein Purification: From Molecular Mechanisms to Large-Scale Processes*, Ladisch, M. R., Willson, R. C., Painton, C. C., and Builder, S. E., eds., American Chemical Society Symposium Series No. 427, Ch 13, 181-193).

Proteases such Factor Xa, thrombin, subtilisin and mutants thereof, have been successfully used to cleave fusion proteins. Typically, a peptide linker that is amenable to cleavage by the protease used is inserted between the "other" protein (e.g., the Z domain of protein A) and the protein of interest, such as humanized anti-IL-8 antibody or antibody fragment. Using recombinant DNA methodology, the nucleotide base pairs encoding the linker are inserted between the genes or gene fragments coding for the other proteins. Proteolytic cleavage of the partially purified fusion protein containing the correct linker can then be carried out on either the native fusion protein, or the reduced or denatured fusion protein.

Various techniques are also available which may now be employed to produce variant humanized antibodies or antibody fragments, which encodes for additions, deletions, or changes in amino acid sequence of the resultant protein(s) relative to the parent humanized antibody or antibody fragment.

By way of illustration, with expression vectors encoding humanized antibody or antibody fragment in hand, site specific mutagenesis (Kunkel *et al.*, *Methods Enzymol.* 204:125-139 (1991); Carter, P., *et al.*, *Nucl. Acids. Res.* **13**:4331 (1986); Zoller, M. J. *et al.*, *Nucl. Acids Res.* **10**:6487 (1982)), cassette mutagenesis (Wells, J. A., *et al.*, *Gene* **34**:315 (1985)), restriction selection mutagenesis (Wells, J. A., *et al.*, *Philos. Trans, R. Soc. London SerA* **317**, 415 (1986))

or other known techniques may be performed on the antibody or antibody fragment DNA. The variant DNA can then be used in place of the parent DNA by insertion into the aforementioned expression vectors. Growth of host bacteria containing the expression vectors with the mutant DNA allows the production of variant humanized antibodies or antibody fragments, which can be isolated as described herein.

## B. Insertion of DNA into a Cloning Vehicle

The DNA encoding the antibody or antibody fragment is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on (1) whether it is to be used for DNA amplification or for DNA expression, (2) the size of the DNA to be inserted into the vector, and (3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

### (i) Signal Sequence Component

In general, a signal sequence may be a component of the vector, or it may be a part of the antibody or antibody fragment DNA that is inserted into the vector. Preferably, a heterologous signal sequence selected and fused to the antibody or antibody fragment DNA such that the signal sequence in the corresponding fusion protein is recognized, transported and processed (*i.e.*, cleaved by a signal peptidase) in the host cell's protein secretion system. In the case of prokaryotic host cells, the signal sequence is selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. In a preferred embodiment, the STII signal sequence is used as described in the Examples below. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader,  $\alpha$  factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders), or acid

phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

5 (ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is homologous to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of the antibody or antibody fragment DNA.

(iii) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, J. Molec. Appl. Genet., 1: 327 (1982)), mycophenolic acid (Mulligan *et al.*, Science, 209: 1422 (1980)) or hygromycin (Sugden *et al.*, Mol. Cell. Biol., 5: 410-413 (1985)). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug (G418 or neomycin (geneticin), xgpt (mycophenolic acid), and hygromycin, respectively.)

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibody or antibody fragment nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the antibody or antibody fragment. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of the antibody or antibody fragment are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the antibody or antibody fragment. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the antibody or antibody fragment, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7. Stinchcomb *et al.*, Nature, 282: 39 (1979); Kingsman *et al.*, Gene, 7: 141 (1979); or Tschemper *et al.*, Gene, 10: 157 (1980). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, Genetics, 85: 12 (1977). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

(iv) Promoter Component

Expression vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody or antibody fragment nucleic acid.

Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as the antibody or antibody fragment encoding sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems (Chang *et al.*, Nature, 275: 615 (1978); and Goeddel *et al.*, Nature, 281: 544 (1979)), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res., 8: 4057 (1980) and EP 36,776) and hybrid promoters such as the tac promoter (deBoer *et al.*, Proc. Natl. Acad. Sci. USA, 80: 21-25 (1983)). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker to operably ligate them to DNA encoding the antibody or antibody fragment (Siebenlist *et al.*, Cell, 20: 269 (1980)) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the antibody or antibody fragment.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, J. Biol. Chem., 255: 2073 (1980)) or other glycolytic enzymes (Hess *et al.*, J. Adv. Enzyme Reg., 7: 149 (1968); and Holland, Biochemistry, 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.

Vector driven transcription of antibody or antibody fragment encoding DNA in mammalian host cells can be controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, Nature, 273: 113 (1978); Mulligan and Berg, Science, 209: 1422-1427 (1980); Pavlakis *et al.*, Proc. Natl. Acad. Sci. USA, 78: 7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway *et al.*, Gene, 18: 355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. 4,419,446. A modification of this system is

described in U.S. 4,601,978. See also Gray *et al.*, Nature, 295: 503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells, Reyes *et al.*, Nature, 297: 598-601 (1982) on expression of human  $\gamma$ -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus, Canaani and Berg, Proc. Natl. Acad. Sci. USA, 79: 5166-5170 (1982) on expression of the human interferon  $\beta$  gene in cultured mouse and rabbit cells, and Gorman *et al.*, Proc. Natl. Acad. Sci. USA, 79: 6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

#### (v) Enhancer Element Component

Transcription of a DNA encoding antibody or antibody fragment by higher eukaryotic host cells is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins *et al.*, Proc. Natl. Acad. Sci. USA, 78: 993 (1981)) and 3' (Lusky *et al.*, Mol. Cell Bio., 3: 1108 (1983)) to the transcription unit, within an intron (Banerji *et al.*, Cell, 33: 729 (1983)) as well as within the coding sequence itself (Osborne *et al.*, Mol. Cell Bio., 4: 1293 (1984)). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297: 17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody or antibody fragment DNA, but is preferably located at a site 5' from the promoter.



(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) can also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the antibody or antibody fragment. The 3' untranslated regions also include transcription termination sites.

Suitable vectors containing one or more of the above listed components and the desired coding and control sequences are constructed by standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, Nucleic Acids Res., 9: 309 (1981) or by the method of Maxam *et al.*, Methods in Enzymology, 65: 499 (1980).

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the antibody or antibody fragment. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the antibody or antibody fragment in recombinant vertebrate cell culture are described in Gething *et al.*, Nature, 293: 620-625 (1981); Mantei *et al.*, Nature, 281: 40-46 (1979); Levinson *et al.*, EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression

of the IgE peptide antagonist is pRK5 (EP pub. no. 307,247) or pSVI6B (PCT pub. no. WO 91/08291 published 13 June 1991).

### C. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacilli* such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescens*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* 1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. In a preferred embodiment, the *E. coli* strain 49D6 is used as the expression host as described in the Examples below. Review articles describing the recombinant production of antibodies in bacterial host cells include Skerra *et al.*, Curr. Opinion in Immunol., 5: 256 (1993) and Pluckthun, Immunol. Revs., 130: 151 (1992).

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for vectors containing antibody or antibody fragment DNA. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *S. pombe* (Beach and Nurse, Nature, 290: 140 (1981)), *Kluyveromyces lactis* (Louvencourt *et al.*, J. Bacteriol., 737 (1983)), *yarrowia* (EP 402,226), *Pichia pastoris* (EP 183,070), *Trichoderma reesia* (EP 244,234), *Neurospora crassa* (Case *et al.*, Proc. Natl. Acad. Sci. USA, 76: 5259-5263 (1979)), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, Biochem. Biophys. Res. Commun., 112: 284-289 (1983); Tilburn *et al.*, Gene, 26: 205-221 (1983); Yelton *et al.*, Proc. Natl. Acad. Sci. USA, 81: 1470-1474 (1984)) and *A. niger* (Kelly and Hynes, EMBO J., 4: 475-479 (1985)).

Host cells derived from multicellular organisms can also be used in the recombinant production of antibody or antibody fragment. Such host cells are capable of complex processing

and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified. See, e.g., Luckow *et al.*, Bio/Technology, 6: 47-55 (1988); Miller *et al.*, in Genetic Engineering, Setlow, J.K. *et al.*, 8: 277-279 (Plenum Publishing, 1986), and Maeda *et al.*, Nature, 315: 592-594 (1985). A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the antibody or antibody fragment DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding antibody or antibody fragment is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the antibody or antibody fragment DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, J. Mol. Appl. Gen., 1: 561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published 21 June 1989.

Vertebrate cell culture is preferred for the recombinant production of full length antibodies. The propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for

growth in suspension culture, Graham *et al.*, J. Gen Virol., 36: 59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23: 243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney  
5 cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci., 383: 44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2).

10 Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells. Myeloma cells that do not otherwise produce immunoglobulin protein are also useful host cells for the recombinant production of full length antibodies.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as  
15 appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO<sub>4</sub> precipitation and electroporation. Successful  
20 transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium  
25 treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, Gene, 23: 315 (1983) and WO 89/05859 published 29 June 1989. For mammalian

cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30-16.37 of Sambrook *et al.*, *supra*, is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, *J. Bact.*, 130: 946 (1977) and Hsiao *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 76: 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or by protoplast fusion may also be used.

#### D. Culturing the Host Cells

Prokaryotic cells used to produce the antibody or antibody fragment are cultured in suitable media as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce the antibody or antibody fragment can be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.*, 58: 44 (1979), Barnes and Sato, *Anal. Biochem.*, 102: 255 (1980), U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. Re. 30,985; or U.S. 5,122,469, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin<sup>TM</sup> drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

#### E. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA, 77: 5201-5205 (1980)), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly <sup>32</sup>P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, Am. J. Clin. Path., 75: 734-738 (1980).

#### F. Purification of the Antibody or Antibody Fragment

In the case of a host cell secretion system, the antibody or antibody fragment is recovered from the culture medium. Alternatively, the antibody can be produced intracellularly, or produced in the periplasmic space of a bacterial host cell. If the antibody is produced intracellularly, as a first step, the host cells are lysed, and the resulting particulate debris is removed, for example, by centrifugation or ultrafiltration. Carter *et al.*, *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human  $\gamma 1$ ,  $\gamma 2$ , or  $\gamma 4$  heavy chains (Lindmark *et al.*, *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human  $\gamma 3$  (Guss *et al.*, *EMBO J.* 5:1567-1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrene-divinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a  $C_H3$  domain, the Bakerbond ABX<sup>TM</sup> resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin Sepharose<sup>TM</sup>, chromatography on an anion

or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

#### G. Production of Antibody Fragments

Various techniques have been developed for the production of the humanized antibody fragments of the invention, including Fab, Fab', Fab'-SH, or F(ab')<sub>2</sub> fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto *et al.*, *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al.*, *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')<sub>2</sub> fragments (Carter *et al.*, *Bio/Technology*, 10:163-167 (1992)). According to another approach, F(ab')<sub>2</sub> fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

### 5. Uses of Anti-IL-8 Antibodies

#### A. Diagnostic Uses

For diagnostic applications requiring the detection or quantitation of IL-8, the antibodies or antibody fragments of the invention typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety can be a radioisotope, such as <sup>3</sup>H, <sup>14</sup>C, <sup>32</sup>P, <sup>35</sup>S, or <sup>125</sup>I; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, e.g., <sup>125</sup>I, <sup>32</sup>P, <sup>14</sup>C, or



<sup>3</sup>H; or an enzyme, such as alkaline phosphatase, beta-galactosidase, or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody or antibody fragment to the detectable moiety can be employed, including those methods described by Hunter *et al.*, Nature 144:945 (1962); David *et al.*, Biochemistry 13:1014 (1974); Pain *et al.*, J. Immunol. Meth. 40:219 (1981); and Nygren, J. Histochem. and Cytochem. 30:407 (1982).

The antibodies and antibody fragments of the present invention can be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. For example, see Zola, Monoclonal Antibodies: A Manual of Techniques, pp. 147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard (which can be a IL-8 or an immunologically reactive portion thereof) to compete with the test sample analyte (IL-8) for binding with a limited amount of antibody or antibody fragment. The amount of IL-8 in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies or antibody fragments generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies can conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different antigenic portion, or epitope, of the protein (IL-8) to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex (U.S. Patent No. 4,376,110). The second antibody can itself be labeled with a detectable moiety (direct sandwich assays) or can be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme (e.g., horseradish peroxidase).

IL-8 antibodies and antibody fragments also are useful for the affinity purification of IL-8 from recombinant cell culture or natural sources. For example, these antibodies can be fixed to a

solid support by techniques well known in the art so as to purify IL-8 from a source such as culture supernatant or tissue.

#### B. Therapeutic Compositions and Administration of Anti-IL-8 Antibody

5 The humanized anti-IL-8 antibodies and antibody fragments of the invention are useful in the treatment of inflammatory disorders, including inflammations of the lung, such as adult respiratory distress syndrome (ARDS) and any stage of acute lung injury in the pathogenesis of ARDS described in Bernard et al., Am. J. Respir. Crit. Care Med., 149: 818-824 (1994), bacterial pneumonia, hypovolemic shock, ischemic reperfusion disorders such as surgical  
10 tissue reperfusion injury, myocardial ischemic conditions such as myocardial infarction, reperfusion after cardiac surgery, cardiac arrest, and constriction after percutaneous transluminal coronary angioplasty, inflammatory bowel disorders such as ulcerative colitis, and autoimmune diseases such as rheumatoid arthritis. In addition, the humanized anti-IL-8 antibodies and antibody fragments of the invention are useful in the treatment of asthmatic diseases, such as  
15 allergic asthma.

Therapeutic formulations of the humanized anti-IL-8 antibodies and antibody fragments are prepared for storage by mixing the antibody or antibody fragment having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, supra), in the form of lyophilized cake or aqueous solutions.

20 Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine,  
25 arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

The humanized anti-IL-8 mAb or antibody fragment to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The humanized anti-IL-8 mAb or antibody fragment ordinarily will be stored in lyophilized form or in solution.

5           Therapeutic humanized anti-IL-8 mAb or antibody fragment compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

10           The route of humanized anti-IL-8 mAb or antibody fragment administration is in accord with known methods, e.g., inhalation, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, by enema or suppository, or by sustained release systems as noted below. Preferably the antibody is given systemically or at a site of inflammation.

15           In one embodiment, the invention provides for the treatment of asthmatic diseases by administration of humanized anti-IL-8 mAb or antibody fragment to the respiratory tract. The invention contemplates formulations comprising humanized anti-IL-8 mAb or antibody fragment for use in a wide variety of devices that are designed for the delivery of pharmaceutical compositions and therapeutic formulations to the respiratory tract. In one aspect, humanized anti-IL-8 mAb or antibody fragment is administered in aerosolized or inhaled form. The humanized anti-IL-8 mAb or antibody fragment, combined with a dispersing agent, or dispersant, can be administered in an aerosol formulation as a dry powder or in a solution or suspension with a diluent.

20           Suitable dispersing agents are well known in the art, and include but are not limited to surfactants and the like. Surfactants are generally used in the art to reduce surface induced aggregation of protein caused by atomization of the solution forming the liquid aerosol.

25           Examples of such surfactants include polyoxyethylene fatty acid esters and alcohols, and polyoxyethylene sorbitan fatty acid esters. Amounts of surfactants used will vary, being generally within the range of about 0.001 to 4% by weight of the formulation. In a specific aspect, the surfactant is polyoxyethylene sorbitan monooleate or sorbitan trioleate.

Liquid aerosol formulations contain the humanized anti-IL-8 mAb or antibody fragment and a dispersing agent in a physiologically acceptable diluent. The dry powder formulations of the invention consist of a finely divided solid form of the humanized anti-IL-8 mAb or antibody fragment and a dispersing agent, and optionally a bulking agent, such as lactose, sorbitol, sucrose, or mannitol, and the like, to facilitate dispersal of the powder. With either the liquid or dry powder aerosol formulation, the formulations must be aerosolized. It must be broken down into liquid or solid particles in order to ensure that the aerosolized dose actually reaches the bronchii and/or alveoli, as desired. For example, in the methods for treatment of asthma provided herein, it is preferable to deliver aerosolized humanized anti-IL-8 mAb or antibody fragment to the bronchii. In other embodiments, such as the present methods for treating ARDS and any stage of acute lung injury in the pathogenesis of ARDS, it is preferable to deliver aerosolized humanized anti-IL-8 mAb or antibody fragment to the alveoli. In general, the mass median dynamic diameter will be 5 micrometers ( $\mu\text{m}$ ) or less to ensure that the drug particles reach the lung bronchii or alveoli (Wearly, L.L., 1991, Crit. Rev. in Ther. Drug Carrier Systems, 8:333).

With regard to construction of the delivery device, any form of aerosolization known in the art, including but not limited to nebulization, atomization or pump aerosolization of a liquid formulation, and aerosolization of a dry powder formulation, can be used in the practice of the invention. A delivery device that is uniquely designed for administration of solid formulations is envisioned. Often, the aerosolization of a liquid or a dry powder formulation will require a propellant. The propellant can be any propellant generally used in the art. Examples of useful propellants include chlorofluorocarbons, hydrofluorocarbons, hydrochlorofluorocarbons, and hydrocarbons, including trifluoromethane, dichlorofluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, and combinations thereof.

In a preferred aspect of the invention, the device for aerosolization is a metered dose inhaler. A metered dose inhaler provides a specific dosage when administered, rather than a variable dose depending on administration. Such a metered dose inhaler can be used with either a liquid or a dry powder aerosol formulation.

Systems of aerosol delivery, such as the pressurized metered dose inhaler and the dry powder inhaler are disclosed in Newman, S.P., *Aerosols and the Lung*, Clarke, S.W. and Davia, D. editors, pp.197-22 and can be used in connection with the present invention.

Sustained release systems can be used in the practice of the methods of the invention.

5 Suitable examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, Biopolymers 22:547 (1983)), poly (2- hydroxyethyl-methacrylate) (Langer *et al.*, J. Biomed. Mater. Res. 15:167 (1981) and Langer, 10 Chem. Tech. 12:98 (1982)), ethylene vinyl acetate (Langer *et al.*, *supra*) or poly-D-(-)-3- hydroxybutyric acid (EP 133,988). Sustained-release humanized anti-IL-8 antibody or antibody fragment compositions also include liposomally entrapped antibody or antibody fragment. Liposomes containing an antibody or antibody fragment are prepared by methods known per se: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. U.S.A. 82:3688 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. U.S.A. 77:4030 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mole percent cholesterol, the selected proportion being adjusted for the most efficacious antibody or antibody fragment therapy.

20 An "effective amount" of the humanized anti-IL-8 antibody or antibody fragment to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. Typically, the clinician will administer the humanized anti-IL-8 25 antibody or antibody fragment until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

In the treatment and prevention of an inflammatory disorder or asthmatic disorder with a humanized anti-IL-8 antibody or antibody fragment of the invention, the antibody composition will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the antibody, the particular type of antibody, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" of antibody to be administered will be governed by such considerations, and is the minimum amount necessary to prevent, ameliorate, or treat the inflammatory disorder, including treating acute or chronic respiratory diseases and reducing inflammatory responses. Such amount is preferably below the amount that is toxic to the host or renders the host significantly more susceptible to infections.

As a general proposition, the initial pharmaceutically effective amount of the antibody or antibody fragment administered parenterally per dose will be in the range of about 0.1 to 50 mg/kg of patient body weight per day, with the typical initial range of antibody used being 0.3 to 20 mg/kg/day, more preferably 0.3 to 15 mg/kg/day.

In one embodiment, using systemic administration, the initial pharmaceutically effective amount will be in the range of about 2 to 5 mg/kg/day.

For methods of the invention using administration by inhalation, the initial pharmaceutically effective amount will be in the range of about 1 microgram ( $\mu$ g)/kg/day to 100 mg/kg/day.

The invention provides for both prophylactic and therapeutic treatment of inflammatory disorders. Without intending to limit the methods of the invention to a particular mechanism of action or a particular disease intervention strategy, it is noted that in some indications it is beneficial to treat the disease in question prior to or early on in the stage of the underlying disease that involves neutrophil activation, recruitment and infiltration at sites of inflammation. Accordingly, it may be advantageous to utilize the humanized anti-IL-8 mAb or antibody fragment in a prophylactic treatment regimen for an inflammatory disease indication in order to

attenuate or eliminate a pathogenic neutrophil response that may or will arise during the course of the disease.

In patients at risk of developing acute lung injury with possible or likely progression to ARDS, it is desirable to employ a prophylactic course of treatment in order to ameliorate or prevent the deterioration of lung function and the pathogenesis of associated disease sequelae (which may greatly increase patient morbidity and mortality) prior to the onset of such conditions. Certain biological parameters, such as IL-8 levels in bronchial alveolar lavage (BAL) fluid and ferritin levels in serum, can be used for prognosis of acute lung injury and ARDS in patients who are predisposed to such disease progression, i.e. patients suffering from diseases or other insults that commonly precipitate acute lung injury and ARDS, such as aspiration, diffuse pulmonary infection, near-drowning, toxic inhalation, lung contusion, multiple trauma, pancreatitis, perforated bowel, sepsis, and the like. In one embodiment, acute lung injury and ARDS at-risk patients presenting BAL fluid IL-8 concentrations of at or above 0.2 ng/ml are selected for prophylactic treatment according to the methods of the invention. Any suitable method for assay of IL-8 in patient BAL fluid may be employed, such as the method described in Donnelly et al., Lancet, 341: 643-647 (1993).

In another embodiment, acute lung injury/ARDS at-risk female and male patients presenting ferritin serum concentrations of at or above 270 ng/ml and 680 ng/ml, respectively, are selected for prophylactic treatment according to the methods of the invention. Any suitable method for assay of ferritin in patient serum may be employed, such as the method described in U.S. Pat. No. 5,679,532 for "Serum Ferritin as a Predictor of the Acute Respiratory Distress Syndrome" to Repine issued on October 21, 1997.

In patients presenting ischemic conditions or undergoing surgical procedures that generate ischemic conditions in tissue and concomitant risk of tissue injury upon reperfusion, it is desirable to employ a course of treatment wherein the humanized anti-IL-8 mAb or antibody fragment is administered to the patient prior to the reperfusion of ischemic tissue, or prior to or as soon as possible after the onset of an inflammatory response following reperfusion of ischemic tissue. In the patients presenting acute myocardial infarction, for example, it is advantageous to

employ a course of treatment wherein the humanized anti-IL-8 mAb or antibody fragment is administered to the patient prior to or concomitant with recanalization therapy, including pharmaceutical recanalization therapies such as the administration of tissue plasminogen activators, streptokinase, or other thrombolytic drugs with or without anti-clotting agents such as platelet-fibrin binding antagonists (e.g. anti-IIbIIIa integrin antibody), blood thinning agents such as heparin, or other anti-reocclusion agents such as aspirin, and the like, and including mechanical recanalization therapies such as percutaneous transluminal coronary angioplasty, or wherein the humanized anti-IL-8 mAb or antibody fragment is administered to the patient prior to or as soon as possible after the onset of an inflammatory response following reperfusion of ischemic myocardium. In yet another embodiment, the humanized anti-IL-8 mAb or antibody fragment of the invention can be employed in the methods of treating acute myocardial infarction with anti-IL-8 antibody described in WO 97/40215 published October 30, 1997.

The invention provides for both prophylactic and therapeutic treatment of asthma with humanized anti-IL-8 mAb and antibody fragment. In the case of prophylactic treatment for allergic asthma with the antibodies or antibody fragments of the invention, it is desirable to administer about 0.1 to 10 mg/kg of the antibody agent to the patient up to about 24 hours prior to anticipated exposure to allergen or prior to onset of allergic asthma. In the case of therapeutic treatment for acute asthma, including allergic asthma, it is desirable to treat the asthmatic patient as early as possible following onset of an asthma attack. In one embodiment, an episode of acute asthma is treated within 24 hours of the onset of symptoms by administration of about 0.1 to 10 mg/kg of an anti-IL-8 antibody agent. However, it will be appreciated that the methods of the invention can be used to ameliorate symptoms at any point in the pathogenesis of asthmatic disease. Additionally, the methods of the invention can be used to alleviate symptoms of chronic asthmatic conditions.

The antibody or antibody fragment need not be, but is optionally formulated with one or more agents currently used to prevent or treat the inflammatory disorder or asthmatic disease in question. For example, in rheumatoid arthritis, the antibody can be given in conjunction with a glucocorticosteroid. In the case of treating asthmatic diseases with anti-IL-8 antibody or



antibody fragment, the invention contemplates the coadministration of antibody or antibody fragment and one or more additional agents useful in treating asthma, such as bronchodilators, antihistamines, epinephrine, and the like. The effective amount of such other agents depends on the amount of antibody or antibody fragment present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all references cited in the specification, and the disclosures of all citations in such references, are expressly incorporated herein by reference.

## EXAMPLES

### A. GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST HUMAN IL-8

Balb/c mice were immunized in each hind footpad or intraperitoneally with 10 µg of recombinant human IL-8 (produced as a fusion of (ser-IL-8)<sub>72</sub> with ubiquitin (Hebert *et al.* J. Immunology 145:3033-3040 (1990)); IL-8 is available commercially from PeproTech, Inc., Rocky Hill, NJ) resuspended in MPL/TDM (Ribi Immunochem. Research Inc., Hamilton, MT) and boosted twice with the same amount of IL-8. In these experiments, "IL-8" is intended to mean (ser-IL-8)<sub>72</sub> unless otherwise specified. A final boost of 10 µg of IL-8 was given 3 days before the fusion. Spleen cells or popliteal lymph node cells were fused with mouse myeloma P3X63Ag8U.1 (ATCC CRL1597), a non-secreting clone of the myeloma P3X63Ag8, using 35% polyethylene glycol as described before. Ten days after the fusion, culture supernatant was screened for the presence of monoclonal antibodies to IL-8 by ELISA.

The ELISA was performed as follows. Nunc 96-well immunoplates (Flow Lab, McLean, VA) were coated with 50 µl/well of 2 µg/ml IL-8 in phosphate-buffered saline (PBS) overnight at 4°C. The remaining steps were carried out at room temperature. Nonspecific binding sites were blocked with 0.5% bovine serum albumin (BSA) for 1 hour (hr). Plates were then

incubated with 50 µl/well of hybridoma culture supernatants from 672 growing parental fusion wells for 1 hr, followed by the incubation with 50 µl/well of 1:1000 dilution of a 1 mg/ml stock solution of alkaline phosphatase-conjugated goat anti-mouse Ig (Tago Co., Foster City, CA) for 1 hr. The level of enzyme-linked antibody bound to the plate was determined by the addition of 100 µl/well of 0.5 mg/ml of p-nitrophenyl phosphate in sodium bicarbonate buffer, pH 9.6. The color reaction was measured at 405 nm with an ELISA plate reader (Titertek Multiscan, Flow Lab, McLean, VA). Between each step, plates were washed three times in PBS containing 0.05% Tween 20.

Culture supernatants which promoted 4-fold more binding of IL-8 than did control medium were selected as positives. According to this criterion, 16 of 672 growing parental fusion wells (2%) were positive. These positive hybridoma cell lines were cloned at least twice by using the limiting dilution technique.

Seven of the positive hybridomas were further characterized as follows. The isotypes of the monoclonal antibodies were determined by coating Nunc 96-well immunoplates (Flow Lab, McLean, VA) with IL-8 overnight, blocking with BSA, incubating with culture supernatants followed by the addition of predetermined amount of isotype-specific alkaline phosphatase-conjugated goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA). The level of conjugated antibodies bound to the plate was determined by the addition of p-nitrophenyl phosphate as described above.

All the monoclonal antibodies tested belonged to either IgG<sub>1</sub> or IgG<sub>2</sub> immunoglobulin isotype. Ascites fluid containing these monoclonal antibodies had antibody titers in the range of 10,000 to 100,000 as determined by the reciprocal of the dilution factor which gave 50% of the maximum binding in the ELISA.

To assess whether these monoclonal antibodies bound to the same epitopes, a competitive binding ELISA was performed. At a ratio of biotinylated mAb to unlabeled mAb of 1:100, the binding of biotinylated mAb 5.12.14 was significantly inhibited by its homologous mAb but not by mAb 4.1.3, while the binding of biotinylated mAb 4.1.3 was inhibited by mAb 4.1.3 but not by mAb 5.12.14. Monoclonal antibody 5.2.3 behaved similarly to mAb 4.1.3, while monoclonal

antibodies 4.8 and 12.3.9 were similar to mAb 5.12.14. Thus, mAb 4.1.3 and mAb 5.2.3 bind to a different epitope(s) than the epitope recognized by monoclonal antibodies 12.3.9, 4.8 and 5.12.14.

Immunodot blot analysis was performed to assess antibody reactivity to IL-8 immobilized on nitrocellulose paper. All seven antibodies recognized IL-8 immobilized on paper, whereas a control mouse IgG antibody did not.

The ability of these monoclonal antibodies to capture soluble  $^{125}\text{I}$ -IL-8 was assessed by a radioimmune precipitation test (RIP). Briefly, tracer  $^{125}\text{I}$ -IL-8 ( $4 \times 10^4$  cpm) was incubated with various dilutions of the monoclonal anti-IL-8 antibodies in 0.2 ml of PBS containing 0.5% BSA and 0.05% Tween 20 (assay buffer) for 1 hr at room temperature. One hundred microliters of a predetermined concentration of goat anti-mouse Ig antisera (Pel-Freez, Rogers, AR) were added and the mixture was incubated at room temperature for 1 hr. Immune complexes were precipitated by the addition of 0.5 ml of 6% polyethylene glycol (M.W. 8000) kept at  $4^\circ\text{C}$ . After centrifugation at  $2,000 \times g$  for 20 min at  $4^\circ\text{C}$ , the supernatant was removed by aspiration and the radioactivity remaining in the pellet was counted in a gamma counter. Percent specific binding was calculated as (precipitated cpm - background cpm) / (total cpm - background cpm). Monoclonal antibodies 4.1.3, 5.2.3, 4.8, 5.12.14 and 12.3.9 captured  $^{125}\text{I}$ -IL-8 very efficiently, while antibodies 9.2.4 and 8.9.1 were not able to capture soluble  $^{125}\text{I}$ -IL-8 in the RIP even though they could bind to IL-8 coated onto ELISA plates (Table I).

The dissociation constants of these monoclonal antibodies were determined using a competitive binding RIP assay. Briefly, competitive inhibition of the binding each antibody to  $^{125}\text{I}$ -IL-8 (20,000-40,000 cpm per assay) by various amounts of unlabeled IL-8 was determined by the RIP described above. The dissociation constant (affinity) of each mAb was determined by using Scatchard plot analysis (Munson, *et al.*, Anal. Biochem. 107:220 (1980)) as provided in the VersaTerm-PRO computer program (Synergy Software, Reading, PA). The  $K_d$ 's of these monoclonal antibodies (with the exception of 9.2.4. and 8.9.1) were in the range from  $2 \times 10^{-8}$  to  $3 \times 10^{-10}$  M. Monoclonal antibody 5.12.14 with a  $K_d$  of  $3 \times 10^{-10}$  M showed the highest affinity among all the monoclonal antibodies tested (Table 3).

Table 3. Characterization of Anti-IL-8 Monoclonal Antibodies

Antibody	%Specific Binding to IL-8	$K_d(M)$	Isotype	pI
4.1.3	58	$2 \times 10^{-9}$	IgG <sub>1</sub>	4.3-6.1
5.2.3	34	$2 \times 10^{-8}$	IgG <sub>1</sub>	5.2-5.6
9.2.4	1	-	IgG <sub>1</sub>	7.0-7.5
8.9.1	2	-	IgG <sub>1</sub>	6.8-7.6
4.8	62	$3 \times 10^{-8}$	IgG <sub>2a</sub>	6.1-7.1
5.12.14	98	$3 \times 10^{-10}$	IgG <sub>2a</sub>	6.2-7.4
12.3.9	86	$2 \times 10^{-9}$	IgG <sub>2a</sub>	6.5-7.1

To assess the ability of these monoclonal antibodies to neutralize IL-8 activity, the amount of <sup>125</sup>I-IL-8 bound to human neutrophils in the presence of various amounts of culture supernatants and purified monoclonal antibodies was measured. Neutrophils were prepared by using Mono-Poly Resolving Medium (M-PRM) (Flow Lab. Inc., McLean, VA). Briefly fresh,

heparinized human blood was loaded onto M-PRM at a ratio of blood to medium, 3.5:3.0, and centrifuged at 300 x g for 30 min at room temperature. Neutrophils enriched at the middle layer were collected and washed once in PBS. Such a preparation routinely contained greater than 95% neutrophils according to the Wright's Giemsa staining. The receptor binding assay was done as follows. 50  $\mu$ l of  $^{125}$ I-IL-8 (5 ng/ml) was incubated with 50  $\mu$ l of unlabeled IL-8 (100  $\mu$ g/ml) or monoclonal antibodies in PBS containing 0.1% BSA for 30 min at room temperature. The mixture was then incubated with 100  $\mu$ l of neutrophils ( $10^7$  cells/ml) for 15 min at 37°C. The  $^{125}$ I-IL-8 bound was separated from the unbound material by loading mixtures onto 0.4 ml of PBS containing 20% sucrose and 0.1% BSA and by centrifugation at 300 x g for 15 min. The supernatant was removed by aspiration and the radioactivity associated with the pellet was counted in a gamma counter.

Monoclonal antibodies 4.1.3, 5.2.3, 4.8, 5.12.14, and 12.3.9 inhibited greater than 85% of the binding of IL-8 to human neutrophils at a 1:25 molar ratio of IL-8 to mAb. On the other hand, monoclonal antibodies 9.2.4 and 8.9.1 appeared to enhance the binding of IL-8 to its receptors on human neutrophils. Since a control mouse IgG also enhanced the binding of IL-8 on neutrophils, the enhancement of IL-8 binding to its receptors by mAb 9.2.4 and 8.9.1 appears to be nonspecific. Thus, monoclonal antibodies, 4.1.3, 5.1.3, 4.8, 5.12.14, and 12.3.9 are potential neutralizing monoclonal antibodies while monoclonal antibodies 8.9.1 and 9.2.4 are non-neutralizing monoclonal antibodies.

The ability of the anti-IL-8 antibodies to block neutrophil chemotaxis induced by IL-8 was tested as follows. Neutrophil chemotaxis induced by IL-8 was determined using a Boyden chamber method (Larsen, *et al.* Science 243:1464 (1989)). One hundred  $\mu$ l of human neutrophils ( $10^6$  cells/ml) resuspended in RPMI containing 0.1% BSA were placed in the upper chamber and 29  $\mu$ l of the IL-8 (20 nM) with or without monoclonal antibodies were placed in the lower chamber. Cells were incubated for 1 hr at 37°C. Neutrophils migrated into the lower chamber were stained with Wright's Giemsa stain and counted under the microscope (100x magnification). Approximately 10 different fields per experimental group were examined. Neutralizing monoclonal antibodies 5.12.14 and 4.1.3 blocked almost 70% of the neutrophil

chemotactic activity of IL-8 at 1:10 ratio of IL-8 to mAb.

The isoelectric focusing (IEF) pattern of each mAb was determined by applying purified antibodies on an IEF polyacrylamide gel (pH 3-9, Pharmacia) using the Fast gel system (Pharmacia, Piscataway, NJ). The IEF gel was pretreated with pharmalyte containing 1% Triton X100 (Sigma, St. Louis, MO) for 10 min before loading the samples. The IEF pattern was visualized by silver staining according to the instructions from the manufacturer. All of the monoclonal antibodies had different IEF patterns, confirming that they originated from different clones. The pI values for the antibodies are listed in Table 3.

All these monoclonal antibodies bound equally well to both (ala-IL-8)<sup>77</sup> and (ser-IL-8)<sup>72</sup> forms of IL-8. Because IL-8 has greater than 30% sequence homology with certain other members of the platelet factor 4 (PF4) family of inflammatory cytokines such as  $\beta$ -TG (Van Damme *et al.*, Eur. J. Biochem. 181:337(1989); Tanaka *et al.*, FEBS 236(2):467 (1988)) and PF4 (Deuel *et al.*, Proc. Natl. Acad. Sci. U.S.A. 74:2256 (1977)), they were tested for possible cross reactivity to  $\beta$ -TG and PF4, as well as to another neutrophil activating factor, C5a. No detectable binding to any of these proteins was observed, with the exception of mAb 4.1.3, which had a slight cross reactivity to  $\beta$ -TG.

One of the antibodies, mAb 5.12.14, was further studied to determine whether it could block the IL-8 mediated release of elastase by neutrophils. Briefly, human neutrophils were resuspended in Hanks balanced salt solution (Gibco, Grand Island, NY) containing 1.0% BSA, Fraction V (Sigma, St. Louis, MO), 2 mg/ml alpha-D-glucose (Sigma), 4.2 mM sodium bicarbonate (Sigma) and 0.01 M HEPES, pH 7.1 (JRH Bioscience, Lenexa, KS). A stock of cytochalasin B (Sigma) was prepared (5 mg/ml in dimethylsulfoxide (Sigma) and stored at 2-8°C. Cytochalasin B was added to the neutrophil preparation to produce a final concentration of 5  $\mu$ g/ml, and incubated for 15 min at 37°C. Human IL-8 was incubated with mAb 5.12.14 (20  $\mu$ l), or a negative control antibody, in 1 ml polypropylene tubes (DBM Scientific, San Fernando, CA) for 30 min at 37°C. The final assay concentrations of IL-8 were 50 and 500 nM. The monoclonal antibodies were diluted to produce the following ratios (IL-8:Mab): 1:50, 1:10, 1:2, 1:1, and 1:0.25. Cytochalasin B-treated neutrophils were added (100  $\mu$ l/tube) and incubated for 2

hours at 25°C. The tubes were centrifuged (210 X g, 2-8°C) for 10 min, and supernatants were transferred to 96 well tissue culture plates (30 µl/well). Elastase substrate stock, 10 mM methoxysuccinyl-alanyl-alanyl-propyl-valyl-p-nitroanilide (Calbiochem, La Jolla, CA) in DMSO was prepared and stored at 2-8°C. Elastase substrate solution (1.2 mM substrate, 1.2 M NaCl (Mallinckrodt, Paris, Kentucky), 0.12 M HEPES pH 7.2 in distilled water) was added (170 µl/well) to the supernatants and incubated for 0.5 to 2 hours at 37°C (until control O.D. of 1.0 was reached). Absorbance was measured at 405 nm (SLT 340 ATTC plate reader, SLT Lab Instruments, Austria).

The results are shown in Figure 1. At a 1:1 ratio of IL-8 to mAb 5.12.14, the antibody was able to effectively block the release of elastase from neutrophils.

The hybridoma producing antibody 5.12.14 was deposited on February 15, 1993 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, U.S.A. (ATCC) and assigned ATTC Accession No. HB 11553.

## B. GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST RABBIT IL-8

Antibodies against rabbit IL-8 were generated in essentially the same process as anti-human IL-8 antibodies using rabbit IL-8 as immunogen (kindly provided by C. Broaddus; see also Yoshimura *et al.* J. Immunol. 146:3483 (1991)). The antibody was characterized as described above for binding to other cytokines coated onto ELISA plates; no measurable binding was found to MGSA, fMLP, C5a, b-TG, TNF, PF4, or IL-1.

The hybridoma producing antibody 6G4.2.5 was deposited on September 28, 1994, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, U.S.A. (ATCC) and assigned ATTC Accession No. HB 11722.

Recombinant human-murine chimeric Fabs for 5.12.14 and 6G4.2.5 were constructed as described below. A chimeric 6G.4.25 Fab is compared with a chimeric 5.12.14 Fab in detail below.

## 1. INHIBITION OF IL-8 BINDING TO HUMAN NEUTROPHILS BY 5.12.14-FAB

### AND 6G4.2.5-FAB

The ability of the two chimeric Fabs, 5.12.14-Fab and 6G4.2.5-Fab, to efficiently bind IL-8 and prevent IL-8 from binding to IL-8 receptors on human neutrophils was determined by performing a competition binding assay which allows the calculation of the  $IC_{50}$  - concentration required to achieve 50% inhibition of IL-8 binding.

Human neutrophils ( $5 \times 10^5$ ) were incubated for 1 hour at  $4^\circ\text{C}$  with  $0.5\text{nM}$   $^{125}\text{I}$ -IL-8 in the presence of various concentrations (0 to 300 nM) of 5.12.14-Fab, 6G4.2.5-Fab, an isotype control (4D5-Fab) or unlabeled IL-8. After the incubation, the unbound  $^{125}\text{I}$ -IL-8 was removed by centrifugation through a solution of 20% sucrose and 0.1% bovine serum albumin in phosphate buffered saline and the amount of  $^{125}\text{I}$ -IL-8 bound to the cells was determined by counting the cell pellets in a gamma counter. Figure 2 demonstrates the inhibition of  $^{125}\text{I}$ -IL-8 binding to neutrophils by unlabeled IL-8. Figure 3 demonstrates that a negative isotype matched Fab does not inhibit the binding of  $^{125}\text{I}$ -IL-8 to human neutrophils. Both the anti-IL-8 Fabs, 5.12.14 Fab (Figure 4) and 6G.4.25 Fab (Figure 5) were able to inhibit the binding of  $^{125}\text{I}$ -IL-8 to human neutrophils with an average  $IC_{50}$  of 1.6 nM and 7.5 nM, respectively.

## 2. INHIBITION OF IL-8-MEDIATED NEUTROPHIL CHEMOTAXIS BY 5.12.14-FAB AND 6G4.2.5-FAB

Human neutrophils were isolated, counted and resuspended at  $5 \times 10^6$  cells/ml in Hank's balanced salt solution (abbreviated HBSS; without calcium and magnesium) with 0.1% bovine serum albumin. The neutrophils were labeled by adding calcein AM (Molecular Probe, Eugene, OR) at a final concentration of  $2.0 \mu\text{M}$ . Following a 30 minute incubation at  $37^\circ\text{C}$ , cells were washed twice with HBSS-BSA and resuspended at  $5 \times 10^6$  cells/ml.

Chemotaxis experiments were carried out in a Neuro Probe (Cabin John, MD) 96-well chamber, model MBB96. Experimental samples (buffer only control, IL-8 alone or IL-8 + Fabs) were loaded in a Polyfiltronics 96-well View plate (Neuro Probe Inc.) placed in the lower chamber.  $100 \mu\text{l}$  of the calcein AM-labeled neutrophils were added to the upper chambers and



allowed to migrate through a 5 micrometer porosity PVP free polycarbonate framed filter (Neuro Probe Inc.) toward the bottom chamber sample. The chemotaxis apparatus was then incubated for 40 to 60 minutes at 37°C with 5% CO<sub>2</sub>. At the end of the incubation, neutrophils remaining in the upper chamber were aspirated and upper chambers were washed three times with PBS.

5 Then the polycarbonate filter was removed, non-migrating cells were wiped off with a squeegee wetted with PBS, and the filter was air dried for 15 minutes.

The relative number of neutrophils migrating through the filter (Neutrophil migration index) was determined by measuring fluorescence intensity of the filter and the fluorescence intensity of the contents of the lower chamber and adding the two values together. Fluorescence intensity was measured with a CytoFluor 2300 fluorescent plate reader (Millipore Corp. Bedford, MA) configured to read a Corning 96-well plate using the 485-20 nm excitation filter and a 530-25 emission filter, with the sensitivity set at 3.

The results are shown in Figures 6 and 7. Figure 6 demonstrates the inhibition of human IL-8 mediated neutrophil chemotaxis by chimeric 6G4.2.5 and 5.12.14 Fabs. Figure 7 demonstrates the relative abilities of chimeric 6G4.2.5 and 5.12.14 Fabs to inhibit rabbit IL-8 mediated neutrophil chemotaxis.

### 3. INHIBITION OF IL-8-MEDIATED NEUTROPHIL ELASTASE RELEASE BY VARIOUS CONCENTRATIONS OF 6G4.2.5 AND 5.12.14 FABS

Blood was drawn from healthy male donors into heparinized syringes. Neutrophils were isolated by dextran sedimentation, centrifugation over Lymphocyte Separation Medium (Organon Teknika, Durham, NC), and hypotonic lysis of contaminating red blood cells as described by Berman *et al.* (*J. Cell Biochem.* 52:183 (1993)). The final neutrophil pellet was suspended at a concentration of  $1 \times 10^7$  cells/ml in assay buffer, which consisted of Hanks Balanced Salt Solution (GIBCO, Grand Island, NY) supplemented with 1.0% BSA (fraction V, Sigma, St. Louis, MO), 2 mg/ml glucose, 4.2 mM sodium bicarbonate, and 0.01 M HEPES, pH 7.2. The neutrophils were stored at 4°C for not longer than 1 hr.

IL-8 (10  $\mu$ l) was mixed with anti-IL-8 Fab, an isotype control Fab, or buffer (20  $\mu$ l) in 1 ml polypropylene tubes and incubated in a 37°C water bath for 30 min. IL-8 was used at final concentrations ranging from 0.01 to 1000 nM in dose response studies (Figure 8) and at a final concentration of 100 nM in the experiments addressing the effects of the Fabs on elastase release (Figures 9 and 10). Fab concentrations ranged from approximately 20 nM to 300 nM, resulting in Fab:IL-8 molar ratios of 0.2:1 to 3:1. Cytochalasin B (Sigma) was added to the neutrophil suspension at a concentration of 5  $\mu$ g/ml (using a 5 mg/ml stock solution made up in DMSO), and the cells were incubated for 15 min in a 37°C water bath. Cytochalasin B-treated neutrophils (100  $\mu$ l) were then added to the IL-8/Fab mixtures. After a 3 hr incubation at room temperature, the neutrophils were pelleted by centrifugation (200 x g for 5 min), and aliquots of the cell-free supernatants were transferred to 96 well plates (30  $\mu$ l/well). The elastase substrate, methoxysuccinyl-alanyl-alanyl-prolyl-valyl-p-nitroanilide (Calbiochem, La Jolla, CA), was prepared as a 10 mM stock solution in DMSO and stored at 4°C. Elastase substrate working solution was prepared just prior to use (1.2 mM elastase substrate, 1.2 M NaCl, 0.12 M HEPES, pH 7.2), and 170  $\mu$ l was added to each sample-containing well. The plates were placed in a 37°C tissue culture incubator for 30 min or until an optical density reading for the positive controls reached at least 1.0. Absorbance was measured at 405 nm using an SLT 340 plate reader (SLT Lab Instruments, Austria).

Figure 9 demonstrates the ability of the chimeric anti-IL-8 Fabs to inhibit elastase release from human neutrophils stimulated by human IL-8; Figure 10 demonstrates the relative abilities of the chimeric anti-IL-8 Fabs to inhibit elastase release from human neutrophils stimulated by rabbit IL-8.

### C. MOLECULAR CLONING OF THE VARIABLE LIGHT AND HEAVY REGIONS OF THE MURINE 5.12.14 (ANTI-IL-8) MONOCLONAL ANTIBODY

Total RNA was isolated from  $1 \times 10^8$  cells (hybridoma cell line ATCC HB-11722) using the procedure described by Chomczynski and Sacchi (*Anal. Biochem.* 162:156 (1987)). First strand cDNA was synthesized by specifically priming the mRNA with synthetic DNA

oligonucleotides designed to hybridize with regions of the murine RNA encoding the constant region of the kappa light chain or the IgG2a heavy chain (the DNA sequence of these regions are published in Sequences of Proteins of Immunological Interest, Kabat, E. A. *et al.* (1991) NIH Publication 91-3242, V 1-3.). Three primers (SEQ ID NOS: 1-6) were designed for each of the

5 light and heavy chains to increase the chances of primer hybridization and efficiency of first strand cDNA synthesis (Figure 13). Amplification of the first strand cDNA to double-stranded (ds) DNA was accomplished using two sets of synthetic DNA oligonucleotide primers: one forward primer (SEQ ID NOS: 7-9) and one reverse primer (SEQ ID NO: 10) for the light chain variable region amplification (Figure 14) and one forward primer (SEQ ID NOS: 11-14) and one

10 reverse primer (SEQ ID NOS: 11, 15, 14 and 13) for the heavy chain variable region amplification (Figure 15). The N-terminal sequence of the first eight amino acids of either the light or heavy chains of 5.12.14 was used to generate a putative murine DNA sequence corresponding to this region. (A total of 29 amino acids was sequenced from the N-terminus of both the light chain and heavy chain variable regions using the Edman degradation protein

15 sequencing technique.) This information was used to design the forward amplification primers which were made degenerate in the third position for some codons to increase the chances of primer hybridization to the natural murine DNA codons and also included the unique restriction site, MluI, for both the light chain variable region forward primer and the heavy chain variable region forward primer to facilitate ligation to the 3' end of the STII element in the cloning vector.

20 The reverse amplification primers were designed to anneal with the murine DNA sequence corresponding to a portion of the constant region of the light or heavy chains near the variable/constant junction. The light chain variable region reverse primer contained a unique BstBI restriction site and the heavy chain variable region reverse primer contained a unique ApaI restriction site for ligation to the 5' end of either the human IgG1 constant light or IgG1 constant

25 heavy regions in the vectors, pB13.1 (light chain) and pB14 (heavy chain). The polymerase chain reaction using these primer sets yielded DNA fragments of approximately 400 bp. The cDNA encoding the 5.12.14 light chain variable region was cloned into the vector pB13.1, to form pA51214VL and the 5.12.14 heavy chain variable region was cloned into the vector, pB14,

to form pA51214VH. The cDNA inserts were characterized by DNA sequencing and are presented in the DNA sequence (SEQ ID NO: 16) and amino acid sequence (SEQ ID NO: 17) of Figure 16 (murine light chain variable region) and in the DNA sequence (SEQ ID NO: 18) and amino acid (SEQ ID NO: 19) of Figure 17 (murine heavy chain variable region).

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#### D. CONSTRUCTION OF A 5.12.14 FAB VECTOR

In the initial construct, pA51214VL, the amino acids between the end of the 5.12.14 murine light chain variable sequence and the unique cloning site, BstBI, in the human IgG1 constant light sequence were of murine origin corresponding to the first 13 amino acids of the murine IgG1 constant region (Figure 16). Therefore, this plasmid contained a superfluous portion of the murine constant region separating the 5.12.14 murine light chain variable region and the human light chain IgG1 constant region. This intervening sequence would alter the amino acid sequence of the chimera and most likely produce an incorrectly folded Fab. This problem was addressed by immediately truncating the cDNA clone after A109 and re-positioning the BstBI site to the variable/constant junction by the polymerase chain reaction. Figure 18 shows the amplification primers used to make these modifications. The forward primer, VL.front (SEQ ID NO: 20), was designed to match the last five amino acids of the STII signal sequence, including the MluI cloning site, and the first 4 amino acids of the 5.12.14 murine light chain variable sequence. The sequence was altered from the original cDNA in the third position of the first two codons D1 (T to C) and I2 (C to T) to create a unique EcoRV cloning site which was used for later constructions. The reverse primer, VL.rear (SEQ ID NO: 21), was designed to match the first three amino acids of the human IgG1 constant light sequence and the last seven amino acids of the 5.12.14 light chain variable sequence which included a unique BstBI cloning site. In the process of adding the BstBI site, the nucleotide sequence encoding several amino acids were altered: L106 (TTG to CTT), K107 (AAA to CGA) resulting in a conservative amino acid substitution to arginine, and R108 (CGG to AGA). The PCR product encoding the modified 5.12.14 light chain variable sequence was then subcloned into pB13.1 in a two-part ligation. The MluI-BstBI digested 5.12.14 PCR product encoding the light chain variable region was ligated

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into MluI-BstBI digested vector to form the plasmid, pA51214VL'. The modified cDNA was characterized by DNA sequencing. The coding sequence for the 5.12.14 light chain is shown in Figure 19.

Likewise, the DNA sequence between the end of the heavy chain variable region and the unique cloning site, ApaI, in the human IgG1 heavy chain constant domain of pA51214VH was reconstructed to change the amino acids in this area from murine to human. This was done by the polymerase chain reaction. Amplification of the murine 5.12.14 heavy chain variable sequence was accomplished using the primers shown in Figure 18. The forward PCR primer (SEQ ID NO: 22) was designed to match nucleotides 867-887 in pA51214VH upstream of the STII signal sequence and the putative cDNA sequence encoding the heavy chain variable region and included the unique cloning site SpeI. The reverse PCR primer (SEQ ID NO: 23) was designed to match the last four amino acids of the 5.12.14 heavy chain variable sequence and the first six amino acids corresponding to the human IgG1 heavy constant sequence which also included the unique cloning site, ApaI. The PCR product encoding the modified 5.12.14 heavy chain variable sequence was then subcloned to the expression plasmid, pMHM24.2.28 in a two-part ligation. The vector was digested with SpeI-ApaI and the SpeI-ApaI digested 5.12.14 PCR product encoding the heavy chain variable region was ligated into it to form the plasmid, pA51214VH'. The modified cDNA was characterized by DNA sequencing. The coding sequence for the 5.12.14 heavy chain is shown in the DNA sequence (SEQ ID NO: 26) and amino acid sequence (SEQ ID NO: 27) of Figures 20A-20B.

The first expression plasmid, pantiIL-8.1, encoding the chimeric Fab of 5.12.14 was made by digesting pA51214VH' with EcoRV and Bpu1102I to replace the EcoRV-Bpu1102I fragment with a EcoRV-Bpu1102I fragment encoding the murine 5.12.14 light chain variable region of pA51214VL'. The resultant plasmid thus contained the murine-human variable/constant regions of both the light and heavy chains of 5.12.14.

Preliminary analysis of Fab expression using pantiIL-8.1 showed that the light and heavy chains were produced intracellularly but very little was being secreted into the periplasmic space of *E. coli*. To correct this problem, a second expression plasmid was constructed.

The second expression plasmid, pantiIL-8.2, was constructed using the plasmid, pmy187, as the vector. Plasmid pantiIL-8.2 was made by digesting pmy187 with MluI and SphI and the MluI (partial)-SphI fragment encoding the murine 5.12.14 murine-human chimeric Fab of pantiIL-8.1 was ligated into it. The resultant plasmid thus contained the murine-human variable/constant regions of both the light and heavy chains of 5.12.14.

The plasmid pantiIL-8.2 was deposited on February 10, 1995 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, U.S.A. (ATCC) and assigned ATCC Accession No. ATCC 97056.

#### E. MOLECULAR CLONING OF THE VARIABLE LIGHT AND HEAVY REGIONS OF THE MURINE 6G4.2.5 MONOCLONAL ANTIBODY

Total RNA was isolated from  $1 \times 10^8$  cells (hybridoma cell line 6G4.2.5) using the procedure described by Chomczynski and Sacchi (Anal. Biochem. 162:156 (1987)). First strand cDNA was synthesized by specifically priming the mRNA with synthetic DNA oligonucleotides designed to hybridize with regions of the murine RNA encoding the constant region of the kappa light chain or the IgG2a heavy chain (the DNA sequence of these regions are published in Sequences of Proteins of Immunological Interest, Kabat *et al.* (1991) NIH Publication 91-3242, V 1-3). Three primers (SEQ ID NOS: 1-6) were designed for each the light and heavy chains to increase the chances of primer hybridization and efficiency of first strand cDNA synthesis (Figure 21). Amplification of the first strand cDNA to double-stranded (ds) DNA was accomplished using two sets of synthetic DNA oligonucleotide primers: one forward primer (SEQ ID NOS: 28-30) and one reverse primer (SEQ ID NO: 31) for the light chain variable region amplification (Figure 22) and one forward primer (SEQ ID NOS: 32-33) and one reverse primer (SEQ ID NOS: 11,15,14 and 13) for the heavy chain variable region amplification (Figure 23). The N-terminal sequence of the first eight amino acids of either the light or heavy chains of 6G4.2.5 was used to generate a putative murine DNA sequence corresponding to this region. (A total of 29 amino acids were sequenced from the N-terminus of both the light chain and heavy chain variable regions using the Edman degradation protein sequencing technique.)

This information was used to design the forward amplification primers which were made degenerate in the third position for some codons to increase the chances of primer hybridization to the natural murine DNA codons and also included the unique restriction site, NsiI, for the light chain variable region forward primer and the unique restriction site, MluI, for the heavy chain variable region forward primer to facilitate ligation to the 3' end of the STII element in the vector, pchimFab. The reverse amplification primers were designed to anneal with the murine DNA sequence corresponding to a portion of the constant region of the light or heavy chains near the variable/constant junction. The light chain variable region reverse primer contained a unique MunI restriction site and the heavy chain variable region reverse primer contained a unique ApaI restriction site for ligation to the 5' end of either the human IgG1 constant light or IgG1 constant heavy regions in the vector, pchimFab. The polymerase chain reaction using these primer sets yielded DNA fragments of approximately 400 bp and were cloned individually into the vector, pchimFab, to form p6G425VL and p6G425VH. The cDNA inserts were characterized by DNA sequencing and are presented in the DNA sequence (SEQ ID NO: 34) and amino acid sequence (SEQ ID NO: 35) of Figure 24 (murine light chain variable region) and the DNA sequence (SEQ ID NO: 36) and amino acid sequence (SEQ ID NO: 37) of Figure 25 (murine heavy chain variable region).

#### F. CONSTRUCTION OF A 6G4.2.5 CHIMERIC FAB VECTOR

In the initial construct, p6G425VL, the amino acids between the end of the 6G4.2.5 murine light chain variable sequence and the unique cloning site, MunI, in the human IgG1 constant light sequence were of murine origin. These amino acids must match the human IgG1 amino acid sequence to allow proper folding of the chimeric Fab. Two murine amino acids, D115 and S121, differed dramatically from the amino acids found in the loops of the  $\beta$ -strands of the human IgG1 constant domain and were converted to the proper human amino acid residues, V115 and F121, by site-directed mutagenesis using the primers (SEQ ID NOS: 38,39,40) shown in Figure 26. These specific mutations were confirmed by DNA sequencing and the modified plasmid named p6G425VL'. The coding sequence is shown in the DNA sequence (SEQ ID NO:

41) and amino acid sequence (SEQ ID NO: 42) of Figures 27A-27B.

Likewise, the DNA sequence between the end of the heavy chain variable region and the unique cloning site, *Apal*, in the human IgG1 heavy chain constant domain of p6G425VH was reconstructed to change the amino acids in this area from murine to human. This process was facilitated by the discovery of a *BstEII* site near the end of the heavy chain variable region. This site and the *Apal* site were used for the addition of a synthetic piece of DNA encoding the corresponding IgG human amino acid sequence. The synthetic oligo-nucleotides shown in Figure 26 were designed as complements of one another to allow the formation of a 27 bp piece of ds DNA. The construction was performed as a three-part ligation because the plasmid, p6G425VH, contained an additional *BstEII* site within the vector sequence. A 5309 bp fragment of p6G425VH digested with *MluI*-*Apal* was ligated to a 388 bp fragment carrying the 6G4.2.5 heavy chain variable region and a 27 bp synthetic DNA fragment encoding the first six amino acids of the human IgG1 constant region to form the plasmid, p6G425VH'. The insertion of the synthetic piece of DNA was confirmed by DNA sequencing. The coding sequence is shown in the DNA sequence (SEQ ID NO: 43) and amino acid sequence (SEQ ID NO: 44) of Figures 28A-28B.

The expression plasmid, p6G425chim2, encoding the chimeric Fab of 6G4.2.5 was made by digesting p6G425chimVL' with *MluI* and *Apal* to remove the STII-murine HPC4 heavy chain variable region and replacing it with the *MluI*-*Apal* fragment encoding the STII-murine 6G4.2.5 heavy chain variable region of p6G425chimVH'. The resultant plasmid thus contained the murine-human variable/constant regions of both the light and heavy chains of 6G4.2.5.

The plasmid p6G425chim2 was deposited on February 10, 1995 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, U.S.A. (ATCC) and assigned ATCC Accession No. 97055.

#### G. CONSTRUCTION OF HUMANIZED VERSIONS OF ANTI-IL-8 ANTIBODY 6G4.2.5

The murine cDNA sequence information obtained from the hybridoma cell line, 6G4.2.5, was used to construct recombinant humanized variants of the murine anti-IL-8 antibody. The



first humanized variant, F(ab)-1, was made by grafting synthetic DNA oligonucleotide primers encoding the murine CDRs of the heavy and light chains onto a phagemid vector, pEMX1 (Werther *et al.*, J. Immunol, 157: 4986-4995 (1996)), which contains a human 6-subgroup I light chain and a human IgG1 subgroup III heavy chain (Fig. 29). Amino acids comprising the framework of the antibody that were potentially important for maintaining the conformations necessary for high affinity binding to IL-8 by the complementarity-determining regions (CDR) were identified by comparing molecular models of the murine and humanized 6G4.2.5 (F(ab)-1) variable domains using methods described by Carter *et al.*, PNAS 89:4285 (1992) and Eigenbrot, *et. al.*, J. Mol. Biol. 229:969 (1993). Additional humanized framework variants (F(ab) 2-9) were constructed from the information obtained from these models and are presented in Table 2 below. In these variants, the site-directed mutagenesis methods of Kunkel, Proc. Natl. Acad. Sci USA, 82:488 (1985) were utilized to exchange specific human framework residues with their corresponding 6G4.2.5 murine counterparts. Subsequently, the entire coding sequence of each variant was confirmed by DNA sequencing. Expression and purification of each F(ab) variant was performed as previously described by Werther *et. al.*, *supra*, with the exception that hen egg white lysozyme was omitted from the purification protocol. The variant antibodies were analyzed by SDS-PAGE, electrospray mass spectroscopy and amino acid analysis.

**Table 4 - Humanized 6G425 Variants**IC50<sup>c</sup>

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Variant	Version	Template	Changes <sup>a</sup>	Purpose <sup>b</sup>	Mean	S.D.	N
F(ab)-1	version 1		CDR Swap		63.0	12.3	4
F(ab)-2	version 2	F(ab)-1	PheH67 <b><i>Ala</i></b>	packaging w/ CDR H2	106.0	17.0	2
F(ab)-3	version 3	F(ab)-1	ArgH71 <b><i>Val</i></b>	packaging w/ CDRs H1, H2	79.8	42.2	4
F(ab)-4	version 6	F(ab)-1	IleH69 <b><i>Leu</i></b>	packaging w/ CDR H2	44.7	9.0	3
F(ab)-5	version 7	F(ab)-1	LeuH78 <b><i>Ala</i></b>	packaging w/ CDRs H1, H2	52.7	31.0	9
F(ab)-6	version 8	F(ab)-1	IleH69 <b><i>Leu</i></b> LeuH78 <b><i>Ala</i></b>	combine F(ab)-4 and -5	34.6	6.7	7
F(ab)-7	version 16	F(ab)-6	LeuH80 <b><i>Val</i></b>	packaging w/ CDR H1	38.4	9.1	2
F(ab)-8	version 19	F(ab)-6	ArgH38 <b><i>Lys</i></b>	packaging w/ CDR H2	14.0	5.7	2
F(ab)-9	version 11	F(ab)-6	GluH6 <b><i>Gln</i></b>	packaging w/ CDR H3	19.0	5.1	7
Chimeric <sup>d</sup> F(ab)					11.4	7.0	1 3
rhu4D5 <sup>e</sup> F(ab)					>200μM		5

- a Amino acid changes made relative to the template used. Murine residues are in bold italics and residue numbering is according to Kabat *et al.*
- b Purpose for making changes based upon interactions observed in molecular models of the humanized and murine variable domains.
- c nM concentration of variant necessary to inhibit binding of iodinated IL-8 to human neutrophils in the competitive binding assay.
- d Chimeric F(ab) is a F(ab) which carries the murine heavy and light chain variable domains fused to the human light chain κI constant domain and the human heavy chain subgroup III constant domain I respectively.
- e. rhu4D5F(ab) is of the same isotype as the humanized 6G425 F(ab)s and is a humanized anti-HER2 F(ab) and therefore should not bind to IL8.

The first humanized variant, F(ab)-1, was an unaltered CDR swap in which all the murine CDR amino acids defined by both x-ray crystallography and sequence hypervariability were transferred to the human framework. When the purified F(ab) was tested for its ability to inhibit <sup>125</sup>I-IL-8 binding to human neutrophils according to the methods described in Section (B)(1) above, a 5.5 fold reduction in binding affinity was evident as shown in Table 4 above.

Subsequent versions of F(ab)-1 were engineered to fashion the 3-dimensional structure of the CDR loops into a more favorable conformation for binding IL-8. The relative affinities of the F(ab) variants determined from competition binding experiments using human neutrophils as described in Section (B)(1) above are presented in Table 4 above. A slight decrease in IL-8 binding (<2 fold) was observed for F(ab)-2-3 while only slight increases in IL-8 binding were noted for F(ab)3-5. Variant F(ab)-6 had the highest increase in affinity for IL-8 (approximately 2 fold), exhibiting an IL-8 binding affinity of 34.6nM compared to the F(ab)-1 IL-8 binding affinity of 63nM. The substitutions of murine Leu for Ile at H69 and murine Ala for Leu at H78 are predicted to influence the packing of CDRs H1 and H2. Further framework substitutions using the F(ab)-6 variant as template were made to bring the binding affinity closer to that of the chimeric F(ab). *In-vitro* binding experiments revealed no change in affinity for F(ab)-7 (38.4nM) but a significant improvement in affinity for F(ab)-8/9 of 14nM and 19 nM, respectively. By analysis of a 3-D computer-generated model of the anti-IL-8 antibody, it was hypothesized that the substitution of murine Lys for Arg at H38 in F(ab)-8 influences CDR-H2 while a change at H6 of murine Gln for Glu in F(ab)-9 affects CDR-H3. Examination of the human antibody sequences with respect to amino acid variability revealed that the frequency of Arg at residue H38 is >99% whereas residue H6 is either Gln ~20% or Glu ~80% (Kabat *et. al.*, Sequences of Proteins of Immunological Interest 5th Ed. (1991)). Therefore, to reduce the likelihood of causing an immune response to the antibody, F(ab)-9 was chosen over F(ab)-8 for further affinity maturation studies. Variant F(ab)-9 was also tested for its ability to inhibit IL-8-mediated chemotaxis (Fig. 30). This antibody was able to block neutrophil migration induced by wild-type human IL-8, human monomeric IL-8 and Rhesus IL-8 with IC<sub>50</sub>'s of approximately 12nM, 15nM, and 22nM, respectively, in IL-8 mediated neutrophil chemotaxis inhibition assays

performed as described in Section (B)(2) above. The amino acid sequence for variant F(ab)-8 is provided in Fig. 31c. The F(ab)-8 was found to block human and rhesus IL-8-mediated chemotaxis with  $IC_{50}$ s of 12nM and 10nM, respectively, in IL-8 mediated neutrophil chemotaxis inhibition assays performed as described in Section (B)(2) above.

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#### H. CONSTRUCTION OF AN ANTI-IL-8-GENE III FUSION PROTEIN FOR PHAGE DISPLAY AND ALANINE SCANNING MUTAGENESIS

An expression plasmid, pPh6G4.V11, encoding a fusion protein (heavy chain of the humanized 6G4.2.5 version 11 antibody and the M13 phage gene-III coat protein) and the light chain of the humanized 6G4.2.5 version 11 antibody was assembled to produce a monovalent display of the anti-IL-8 antibody on phage particles. The construct was made by digesting the plasmid, pFPHX, with EcoRV and ApaI to remove the existing irrelevant antibody coding sequence and replacing it with a 1305bp EcoRV-ApaI fragment from the plasmid, p6G4.V11, encoding the humanized 6G4.2.5 version 11 anti-IL-8 antibody. The translated sequence of the humanized 6G4.2.5 version 11 heavy chain (SEQ ID NO: 52), peptide linker and gene III coat protein (SEQ ID NO: 53) is shown in Fig. 31A. The pFPHX plasmid is a derivative of pHGHam-3 which contains an in-frame amber codon (TAG) between the human growth hormone and gene-III DNA coding sequences. When transformed into an amber suppressor strain of *E. coli*, the codon (TAG) is read as Glutamate producing a growth hormone (hGH)-gene III fusion protein. Likewise, in a normal strain of *E. coli*, the codon (TAG) is read as a stop preventing translational read-through into the gene-III sequence and thus allowing the production of soluble hGH. The pHGHam-3 plasmid is described in Methods: A Companion to Methods in Enzymology, 3:205 (1991). The final product, pPh6G4.V11, was used as the template for the alanine scanning mutagenesis of the CDRs and for the construction of randomized CDR libraries of the humanized 6G4.V11 antibody.

I. ALANINE SCANNING MUTAGENESIS OF HUMANIZED ANTIBODY 6G4.2.5  
VERSION 11

The solvent exposed amino acid residues in the CDRs of the humanized anti-IL-8 6G4.2.5 version 11 antibody (h6G4V11) were identified by analysis of a 3-D computer-generated model of the anti-IL-8 antibody. In order to determine which solvent exposed amino acids in the CDRs affect binding to interleukin-8, each of the solvent exposed amino acids was individually changed to alanine, creating a panel of mutant antibodies wherein each mutant contained an alanine substitution at a single solvent exposed residue. The alanine scanning mutagenesis was performed as described by Leong *et. al.*, J. Biol. Chem., 269: 19343 (1994)).

The IC<sub>50</sub>'s (relative affinities) of h6G4V11 wt and mutated antibodies were established using a Competition Phage ELISA Assay described by Cunningham *et. al.*, (EMBO J. 13:2508 (1994)) and Lee *et. al.*, (Science 270:1657 (1995)). The assay measures the ability of each antibody to bind IL-8 coated onto a 96-well plate in the presence of various concentrations of free IL-8 (0.2 to 1uM) in solution. The first step of the assay requires that the concentrations of the phage carrying the wild type and mutated antibodies be normalized, allowing a comparison of the relative affinities of each antibody. The normalization was accomplished by titering the phage on the IL-8 coated plates and establishing their EC<sub>50</sub>. Sulfhydryl coated 96-well binding plates (Corning-Costar; Wilmington, MA) were incubated with a 0.1mg/ml solution of K64C IL-8 (Lysine 64 is substituted with Cysteine to allow the formation of a disulfide bond between the free thiol group of K64C IL-8 and the sulfhydryl coated plate, which results in the positioning of the IL-8 receptor binding domains towards the solution interface) in phosphate buffered saline (PBS) pH 6.5 containing 1mM EDTA for 1 hour at 25°C followed by three washes with PBS and a final incubation with a solution of PBS containing 1.75mg/ml of L-cysteine-HCl and 0.1M NaHCO<sub>3</sub> to block any free reactive sulfhydryl groups on the plate. The plates were washed once more and stored covered at 4°C with 200ul of PBS/well. Phage displaying either the reference antibody, h6G4V11, or the mutant h6G4V11 antibodies were grown and harvested by PEG precipitation. The phage were resuspended in 500ul 10mM Tris-HCl pH 7.5, 1mM EDTA and 100mM NaCl and held at 4°C for no longer than 3 hours. An aliquot of each phage was diluted

4-fold in PBS containing 0.05% Tween-20 (BioRad, Richmond, Ca.) and 0.5% BSA RIA grade (Sigma, St. Louis, Mo.) (PBB) and added to IL-8 coated plates blocked for at least 2 hours at 25°C with 50mg/ml skim milk powder in 25mM Carbonate Buffer pH 9.6. The phage were next serially diluted in 3 fold steps down the plate from well A through H. The plates were incubated for 1 hour at 25°C followed by nine quick washes with PBS containing 0.05% Tween-20 (PBST). The plates were then incubated with a 1:3200 dilution of rabbit anti-phage antibody and a 1:1600 dilution of secondary goat-anti-rabbit Fc HRP-conjugated antibody for 15 minutes at 25°C followed by nine quick washes with PBST. The plates were developed with 80ul/well of 1mg/ml OPD (Sigma, St. Louis, Mo) in Citrate Phosphate buffer pH 5.0 containing 0.015%  $H_2O_2$  for 4 minutes at 25°C and the reaction stopped with the addition of 40ul of 4.5M  $H_2SO_4$ . The plates were analyzed at wavelength  $8_{492}$  in a SLT model 340ATTC plate reader (SLT Lab Instruments). The individual  $EC_{50}$ s were determined by analyzing the data using the program Kaleidagraph (Synergy Software, Reading, Pa.) and a 4-parameter fit equation. The phage held at 4°C were then immediately diluted in PBB to achieve a final concentration corresponding to their respective  $EC_{50}$  or target  $OD_{492}$  for the competition segment of the experiment, and dispensed into a 96 well plate containing 4-fold serial dilutions of soluble IL-8 ranging from 1uM in well A and ending with 0.2uM in well H. Using a 12-channel pipet, 100ul of the phage/IL-8 mixture was transferred to an IL-8 coated 96-well plate and executed as described above. Each sample was done in triplicate - 3 columns/sample.

**Table 5 - Relative Affinities (IC50) for Alanine-scan Anti-IL-8 6G4V11 CDR Mutants**

CDR	Amino Acid Residue	Avg IC50 (nM)	Std Dev
V11	Reference	11.5	6.4
CDR-L1	S26	6.3	2.9
	Q27	10.2	2.4
	S28	14.2	5.2
	V30	29.1	12.3
	H31	580.3	243.0
	I33	64.2	14.6
	N35	3.3	0.7
	T36	138.0	nd
	Y37	NDB	nd
CDR-L2	K55	24.2	14.9
	V56	15.5	3.8
	S57	12.4	4.0
	N58	17.6	3.7
	R59	nd	nd
CDR-L3	S96	10.8	4.4
	T97	70.6	55.2
	H98	8.0	1.2
	V99	19.6	1.9

CDR	Amino Acid Residue	Avg IC50 (nM)	Std Dev
CDR-H1	S28	8.6	3.1
	S30	nd	nd
	S31	7.8	2.5
	H32	13.3	5.8
	Y53	48.2	15.8
CDR-H2	Y50	35.6	13.0
	D52	13.3	7.5
	S53	6.0	3.4
	N54	96.0	5.8
	E56	15.8	4.5
	T57	8.4	1.6
	T58	11.3	1.8
	Y59	9.1	3.7
	Q61	12.6	6.4
	K64	18.5	12.1
CDR-H3	D96	NDB	nd
	Y97	NDB	nd
	R98	36.6	15.3
	Y99	199.5	nd
	N100	278.3	169.4



CDR	Amino Acid Residue	Avg IC50 (nM)	Std Dev
	D102	159.2	44
	W103	NDB	nd
	F104	NDB	nd
	F105	209.4	72.3
	D106	25.3	21.7

Each sample performed in triplicate/experiment.

NDB = No Detectable Binding /nd = value not determined\*

Residue numbering is according to Kabat et al.

The results of the alanine-scan are summarized in Table 5 above. The alanine substitutions in of many of the mutant antibodies had little or no adverse effects (<3 fold) on the binding affinity for IL-8. Mutants that were found to exhibit no detectable binding of IL-8 (NDB) presumably contained disruptions in the conformational structure of the antibody conferred by crucial structural or buried amino acids in the CDR. Based on the results of the scan, CDR-H3 (heavy chain, 3rd CDR) was identified as the dominant binding epitope for binding IL-8. Alanine substitutions in this CDR resulted in a 3 to >26 fold decrease in binding affinity. The amino acids, Y597, Y599 and D602 are of particular interest because it was determined from the computer generated model of the anti-IL-8 antibody that these residues are solvent exposed and that these residues might participate in hydrogen bonding or charge interactions with IL-8 or other amino acids of the antibody that influence either binding to IL-8 or the conformation of the CDR-H3 loop structure. (See the model depicted in Fig. 32). Unexpected increases in binding affinity (1.8 > 2.7 fold) were noted for S528 and S531 of CDR-H1 and S553 of CDR-H2.

Surprisingly, a significant increase in binding affinity was observed in the alanine mutant N35A located in CDR-L1 (light chain, 1st CDR). A 3-6 fold increase in affinity was observed compared to the wild-type h6G4V11 antibody. This augmentation of IL-8 binding could be the result of the close proximity of N35A to CDR-H3. The alanine substitution may have imparted a

slight change in the conformation of CDR-L1 which alters the packing interaction of neighboring amino acid residues on CDR-H3, thereby tweaking the loop of CDR-H3 into a conformation that facilitates more appropriate contacts with IL-8. Similarly, N35A may also influence the orientation of amino acids in CDR-L1 or its interaction directly with IL-8. Unexpected increases in affinity (~2 fold) were also observed for S26 of CDR-L1 and H98 of CDR-L3.

#### J. CHARACTERIZATION OF HUMANIZED ANTI-IL-8 ANTIBODY 6G4V11N35A

Soluble 6G4V11N35A Fab antibody was made by transforming an amber non-suppressor strain of *E. coli*, 34B8, with pPh6G4.V11 and growing the culture in low phosphate medium for 24 hours. The periplasmic fraction was collected and passed over a Hi-Trap Protein-G column (Pharmacia, Piscataway, NJ.) followed by a desalting and concentration step. The protein was analyzed by SDS-PAGE, mass spectrometry and amino acid analysis. The protein had the correct size and amino acid composition (Fig. 35). The 6G4V11N35A Fab was tested for its ability to inhibit <sup>125</sup>I-IL-8 binding to human neutrophils and to inhibit IL-8 mediated neutrophil chemotaxis as described in Section (B)(1) and (B)(2) above. As shown in Fig. 33, hybridoma-derived intact murine antibody (6G4 murine mAB), recombinant 6G4 murine-human chimera Fab, recombinant humanized Fab versions 1 and 11, and 6G4V11N35A Fab were found to inhibit <sup>125</sup>I-IL-8 binding to human neutrophils with an average IC<sub>50</sub> of 5nM, 8nM, 40nM, 10nM and 3nM, respectively. The 6G4V11N35A Fab had at least a 2-fold higher affinity than the 6G4.2.5 chimera Fab and a 3-fold higher affinity than 6G4V11. As shown in Fig. 34, the 6G4V11N35A Fab was found to inhibit IL-8 mediated neutrophil chemotaxis induced by both wild type and monomeric human IL-8, and by two different animal species of IL-8, namely, rabbit and rhesus. The irrelevant isotype control Fab (4D5) did not inhibit neutrophil migration. The average IC<sub>50</sub> values were 3nM (wt IL-8), 1 nM (monomeric IL-8), 5nM (Rabbit IL-8), and 10nM (Rhesus IL-8).

K. CONSTRUCTION OF A 6G4V11N35A F(ab')<sub>2</sub> LEUCINE ZIPPER

Production of a F(ab')<sub>2</sub> version of the humanized anti-IL-8 6G4V11N35A Fab was accomplished by constructing a fusion protein with the yeast GCN4 leucine zipper. The expression plasmid p6G4V11N35A.F(ab')<sub>2</sub> was made by digesting the plasmid p6G425chim2.fab2 with the restriction enzymes bsaI and apaI to remove the DNA sequence encoding the 6G4.2.5 murine-human chimeric Fab and replacing it with a 2620bp bsaI-apaI fragment from pPh6G4.V11N35A. The plasmid p6G425chim2.fab2 is a derivative of pS1130 which encodes a fusion protein (the GCN4 leucine zipper fused to the heavy chain of anti-CD18) and the light chain of anti-CD18 antibody. The expression plasmid p6G4V11N35A.F(ab')<sub>2</sub> was deposited on February 20, 1996 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, U.S.A. (ATCC) and assigned ATCC Accession No. 97890. A pepsin cleavage site in the hinge region of the antibody facilitates the removal of the leucine zipper leaving the two immunoglobulin monomers joined by the cysteines that generate the interchain disulfide bonds. The DNA and protein sequence of the h6G4V11N35A.F(ab')<sub>2</sub> are depicted in Figs. 35-37.

An expression host cell was obtained by transforming E. coli strain 49D6 with p6G4V11N35A.F(ab')<sub>2</sub> essentially as described in Section (II)(3)(C) above. The transformed host E. coli 49D6 (p6G4V11N35A.F(ab')<sub>2</sub>) was deposited on February 20, 1997 at the ATCC and assigned ATCC Accession No. 98332. Transformed host cells were grown in culture, and the 6G4V11N35A F(ab')<sub>2</sub> product was harvested from the host cell periplasmic space essentially as described in Section (II)(3)(F) above.

L. CHARACTERIZATION OF THE HUMANIZED 6G4V11N35A F(ab')<sub>2</sub> LEUCINE ZIPPER

The 6G4V11N35A Fab and F(ab')<sub>2</sub> were tested for their ability to inhibit <sup>125</sup>I-IL-8 binding to neutrophils according to the procedures described in Section (B)(1) above. The displacement curves from a representative binding experiment performed in duplicate is depicted in Fig. 38. Scatchard analysis of this data shows that 6G4V11N35A F(ab')<sub>2</sub> inhibited <sup>125</sup>I-IL-8 binding to human neutrophils with an average IC<sub>50</sub> of 0.7 nM (+/- 0.2). This is at least a 7 fold increase in

affinity compared to the hybridoma-derived intact murine antibody (average  $IC_{50}$  of 5 nM) and at least a 2.8 fold increase in affinity over the Fab version (average  $IC_{50}$  of 2 nM).

The 6G4V11N35A F(ab')<sub>2</sub> was also tested for its ability to inhibit IL-8 mediated neutrophil chemotaxis according to the procedures described in Section (B)(2) above. The results of a representative chemotaxis experiment performed in quadruplicate are depicted in Fig. 39. As shown in Fig. 39, the 6G4V11N35A F(ab')<sub>2</sub> inhibited human IL-8 mediated neutrophil chemotaxis. The 6G4V11N35A F(ab')<sub>2</sub> exhibited an average  $IC_{50}$  value of 1.5nM versus 2.7nM for the 6G4V11N35A Fab, which represents an approximately 2 fold improvement in the antibody's ability to neutralize the effects of IL-8. The irrelevant isotype control Fab (4D5) did not inhibit neutrophil migration. Furthermore, the 6G4V11N35A F(ab')<sub>2</sub> antibody retained its ability to inhibit IL-8 mediated neutrophil chemotaxis by monomeric IL-8 and by two different animal species of IL-8, namely rabbit and rhesus, in neutrophil chemotaxis experiments conducted as described above. An individual experiment is shown in Fig. 40. The average  $IC_{50}$  values were 1nM (monomeric IL-8), 4nM (Rabbit IL-8), and 2.0nM (Rhesus IL-8).

#### M. RANDOM MUTAGENESIS OF LIGHT CHAIN AMINO ACID (N35A) IN CDR-L1 OF HUMANIZED ANTIBODY 6G4V11

A 3-fold improvement in the  $IC_{50}$  for inhibiting <sup>125</sup>I-IL-8 binding to human neutrophils was observed when alanine was substituted for asparagine at position 35 in CDR-L1 (light chain) of the humanized 6G4V11 mAb as described in Section (I) above. This result might be attributed to an improvement in the contact between the antigen-antibody binding interfaces as a consequence of the replacement of a less bulky nonpolar side chain (R-group) that may have altered the conformation of CDR-L1 or neighboring CDR-H3 (heavy chain) to become more accessible for antigen docking. The acceptance of alanine at position 35 of CDR-L1 suggested that this position contributed to improved affinity and that an assessment of the re-modeling of CDR loops / antigen-binding region(s) by other amino acids at this location was warranted. Selection of an affinity matured version of the humanized 6G4.V11 mAB (Kunkel, T. A., Proc. Natl. Acad. Sci. USA, 82:488 (1995)) was accomplished by randomly mutagenizing position 35 of CDR-L1 and constructing an antibody-phage library. The codon

for Asparagine (N) at position 35 of CDR-L1, was targeted for randomization to any of the 20 known amino acids.

Initially, a stop template, pPh6G4.V11-stop, was made to eliminate contaminating wild-type N35 sequence from the library. This was accomplished by performing site-directed mutagenesis (Muta-Gene Kit, Biorad, Richmond, CA) of pPH6G4V11 (described in Section (H) above) to replace the codon (AAC) for N35 with a stop codon (TAA) using the primer SL.97.2 (SEQ ID NO:63 )(Figure 42). The incorporation of the stop codon was confirmed by DNA sequencing. Subsequently, uracil containing single-stranded DNA derived from *E. coli* CJ236 transformed with the stop template was used to generate an antibody-phage library following the method described by Lowman (Methods in Molecular Biology, 87 Chapter 25: 1-15 (1997)). The variants generated from this library were predicted to produce a collection of antibodies containing one of the 20 known amino acids at position N35 in CDR-L1. The amino acid substitutions were accomplished by site-directed mutagenesis using the degenerate oligonucleotide primer (SL.97.3) with the sequence NNS (N = A/G/T/C ; S = G/C; ) (SEQ ID NO: 64)(Figure 42). This codon usage should allow for the expression of any of the 20 amino acids - including the amber stop codon (TAG). The collection of antibody-phage variants was transfected into *E. coli* strain XL-1 blue (Stratagene, San Diego, CA) by electroporation and grown at 37°C overnight to amplify the library. Selection of tight binding humanized 6G4V11 Fab's were accomplished by panning the library on IL-8 coated 96-well plates as described in Section (I) above. Prior to panning, the number of phage/library was normalized to  $1.1 \times 10^{13}$  phage/ml (which produces a maximum OD<sub>270</sub> reading = 1 OD unit) and IL-8 coated plates were incubated with blocking solution (25mN Carbonate buffer containing 50mg/ml skim milk) for 2 hours before the addition of phage (each sort used eight IL-8 coated wells/library). After the blocking and washing steps, every sort began with the addition of 100ul of antibody-phage (titered at  $1.1 \times 10^{13}$  phage/ml) to each of eight IL-8 coated wells followed by an 1 hour incubation at 25°C. The non-specifically bound antibody-phage were removed by 10 quick washes with PBS-0.05% Tween 20 (PBS-Tween). For sort #1, a low stringency wash (100ul PBS-Tween/well for 10 minutes at 25°C) was employed to capture the small proportion of tight binding antibody-phage bound to the immobilized IL-8. The antibody-phage variants specifically bound to IL-8 were eluted with 100ul/well of 200mM Glycine pH 2.0 for 5 minutes at 25°C. The eluted antibody-

phage variants from the 8 wells were then pooled and neutralized with 1M Tris-HCl pH 8.0 (1/3 the elution volume). The phage were titered and propagated as described in Section (I) above. The stringency of the washes were successively increased with each round of panning depending upon the percent recovery of phage at the end of a sort. The wash conditions were as follows: sort #2 (4 x 15 minute intervals; total time = 60 minutes) and sort #3 (either #3a: 8 x 15 minute intervals or #3b: 12 x 10 minute intervals; total time = 120 minutes). The total number of phage recovered was progressively reduced after each sort suggesting that non- or weak- binders were being selected against. The recovery of the negative control (the antibody-phage stop variant) was constant throughout the panning (approximately 0.0001 to 0.00001 percent).

Eighteen random variants from sort #3 were analyzed by DNA sequencing to look for an amino acid consensus at position 35 of CDR-L1. The data presented in Figure 43A showed that Glycine occupied position 35 in 33% of the variants sequenced. However, after correcting for the number of NNS codon combinations/amino acid, the frequency of Glycine was reduced to 16.6%. Glutamic Acid was represented with the highest frequency (22%) followed by Aspartic Acid and Glycine (16.6%). The frequencies of recovery of the wild-type Asparagine and substituted Alanine were only 5.6%. Interestingly, the high frequency of Glycine may suggest that a much wider range of conformations might be allowed for the loop of CDR-L1 which may be attributed to the reduction in steric hindrance of bond angle ( $\phi$ - $\psi$ ) pairing as a result of the single hydrogen atom as the side chain. Conversely, Glutamic Acid at position 35 might restrict the flexibility of the loop by imposing less freedom of rotation imposed by the more rigid and bulky charged polar side chain.

Soluble Fab's of the affinity matured variants (N35G, N35D, N35E and N35A) were made as described in Section (J) above for evaluating their ability to block IL-8 binding. As shown in Figure 43B, variants N35A, N35D, N35E and N35G were found to inhibit <sup>125</sup>I-IL-8 binding to human neutrophils with an approximate IC<sub>50</sub> of 0.2nM, 0.9nM, 0.1nM and 3.0nM, respectively. All of the affinity matured variants showed an improvement in binding IL-8 ranging from 3 - 100 fold compared to the humanized 6G4V11 mAb. The affinity-matured variant, 6G4V11N35E, was 2-fold more potent in blocking IL-8 binding to human neutrophils than the alanine-scan variant, 6G4V11N35A.

Equilibrium and kinetic measurements of variants 6G4V11N35A and 6G4V11N35E were

determined using KinEXA™ automated immunoassay system (Sapidyne Instruments Inc., Idaho City, ID) as described by Blake et al., *J. Biol. Chem.* 271: 27677 (1996). The procedure for preparing the antigen-coated particles was modified as follows: 1 ml of activated agarose beads (Reacti-Gel 6X; Pierce, Rockford, IL ) were coated with antigen in 50mM Carbonate buffer pH 9.6 containing 20ug/ml of human IL-8 and incubated with gentle agitation on a rocker overnight at 25°C. The IL-8 coated beads were then washed twice with 1M Tris-HCl pH 7.5 to inactivate any unreactive groups on the beads and blocked with Superblock (Pierce, Rockford, IL) for 1 hour at 25°C to reduce non-specific binding. The beads were resuspended in assay buffer (0.1% bovine serum albumin in PBS) to a final volume of 30 ml. A 550ul aliquot of the IL-8 coated bead suspension was used each time to pack a fresh 4mm high column in the KinEXA observation cell. The amount of unbound antibody from the antibody-antigen mixtures captured by the IL-8-coated beads in both the equilibrium and kinetic experiments was quantified using a fluorescently labeled secondary antibody. Murine 6G4.2.5 was detected with a R-PE AffiniPure F(ab')<sub>2</sub> goat anti-mouse IgG, Fc fragment specific 2° antibody (Jackson Immuno Research Laboratories, West Grove, PA) and humanized affinity matured N35A (Fab and F(ab')<sub>2</sub>) and N35E Fab were detected with a R-PE AffiniPure F(ab')<sub>2</sub> donkey anti-human IgG (H+L) 2° antibody (Jackson Immunoresearch Laboratories, West Grove, PA ) ; both at a 1:1000 dilution.

Equilibrium measurements were determined by incubating a constant amount of anti-IL-8 antibody (0.005ug/ml) with various concentrations of human IL-8 (0, 0.009, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5nM ). The antibody-antigen mixture was incubated for 2 hours at 25°C to allow the molecules to reach equilibrium. Subsequently, each sample was passed over a naive IL-8 coated bead pack in the KinEXA observation cell at a flow rate of 0.5ml/minute for a total of 9 minutes/sample. The equilibrium constant (Kd) was calculated using the software provided by Sapidyne Instruments Inc.

Rates of association (ka) and dissociation (kd) were determined by incubating together a constant amount of antibody and antigen, and measuring the amount of uncomplexed anti-IL-8 bound to the IL-8 coated beads over time. The concentration of antibody used in the kinetic experiments was identical to that used in the equilibrium experiment described above. Generally, the amount of human IL-8 used was the concentration derived from the binding curves of the equilibrium experiment that resulted in 70% inhibition of anti-IL-8 binding to the IL-8 coated beads. Measurements were made every 15

minutes to collect approximately nine data points. The  $k_a$  was calculated using the software provided by Sapidyne Instruments, Inc. The off rate was determined using the equation:  $k_d = K_d/k_a$ .

Figure 44 shows the equilibrium constants ( $K_d$ ) for the affinity matured variants 6G4V11N35E and 6G4V11N35A Fab's were approximately 54pM and 114pM, respectively. The improvement in affinity of 6G4V11N35E Fab for IL-8 can be attributed to a 2-fold faster rate of association ( $K_{on}$ ) of  $4.7 \times 10^6$  for 6G4V11N35E Fab versus  $2.0 \times 10^6$  for 6G4V11N35A F(ab')<sub>2</sub>. (The  $K_d$  of the 6G4V11N35A F(ab')<sub>2</sub> and 6G4V11N35A Fab are similar.) The dissociation rates ( $K_{off}$ ) were not significantly different. Molecular modeling suggests that substitution of Asparagine with Glutamic Acid might either affect the antibody's interaction with IL-8 directly or indirectly by neutralizing the charge of neighboring residues R98 (CDR-H3) or K50 (CDR-L2) in the CDR's to facilitate contact with IL-8. Another effect might be the formation of a more stable loop conformation for CDR-L1 that could have facilitated more appropriate contacts of other CDR-L1 loop residues with IL-8. The DNA (SEQ ID NO: 65) and amino acid (SEQ ID NO:62) sequences of p6G4V11N35E.Fab showing the Asparagine to Glutamic Acid substitution in the light chain are presented in Figure 45.

#### N. CHARACTERIZATION OF HUMANIZED ANTI-IL-8 VARIANT 6G4V11N35E Fab

The affinity matured Fab variant, 6G4V11N35E, was tested for its ability to inhibit IL-8 mediated neutrophil chemotaxis as described in Section (B)(2) above. The reuseable 96-well chemotaxis chamber described in Section (B)(2) was replaced with endotoxin-free disposable chemotaxis chambers containing 5-micron PVP-free polycarbonate filters (ChemoTx101-5, Neuro Probe, Inc. Cabin John, MD). As illustrated in Figure 46, variant N35E effectively blocks IL-8 mediated neutrophil chemotaxis induced by a 2nM stimulus of either rabbit or human IL-8. In fact, the level of inhibition at antibody concentrations between 3.7nM - 33nM was not significantly different from the buffer control indicating variant N35E could completely inhibit this response. The  $IC_{50}$ 's for both rabbit and human IL-8 were approximately 2.8nM and 1.2nM, respectively. The irrelevant isotype control Fab (4D5) did not inhibit neutrophil migration indicating the results observed for the affinity matured variant, N35E, is IL-8 specific.



O. CONSTRUCTION OF HUMANIZED 6G4V11N35E F(ab')<sub>2</sub> LEUCINE ZIPPER

A F(ab')<sub>2</sub> expression plasmid for 6G4V11N35E was constructed using methods similar to those described in Section (K) above. The expression plasmid, p6G4V11N35E.F(ab')<sub>2</sub>, was made by digesting the plasmid p6G4V11N35A.F(ab')<sub>2</sub> (described in Section (K) above) with the restriction enzymes ApaI and NdeI to isolate a 2805 bp fragment encoding the heavy chain constant domain - GCN4 leucine zipper and ligating it to a 3758 bp ApaI-NdeI fragment of the pPH6G4V11N35E phage display clone (encoding 6G4V11N35E Fab) obtained as described in Section (M) above. The integrity of the entire coding sequence was confirmed by DNA sequencing.

P. CONSTRUCTION OF THE FULL LENGTH HUMANIZED 6G4V11N35A IgG EXPRESSION PLASMID

The full length IgG<sub>1</sub> version of the humanized anti-IL8 variant 6G4V11N35A was made using a dicistronic DHFR-Intron expression vector (Lucas et al., *Nucleic Acids Res.*, 24: 1774-1779 (1996)) which contained the full length recombinant murine-human chimera of the 6G4.2.5 anti-IL8 mAb. The expression plasmid encoding the humanized variant 6G4V11N35A was assembled as follows. First an intermediate plasmid (pSL-3) was made to shuttle the sequence encoding the variable heavy chain of humanized anti-IL-8 variant 6G4V11N35A to pRK56G4chim.2Vh - which contains the variable heavy region of the chimeric 6G4.5 anti-IL8 antibody. The vector pRK56G4chim.Vh was digested with PvuII and ApaI to remove the heavy chain variable region of the chimeric antibody and religated with an 80bp PvuII - XhoI synthetic oligonucleotide (encoding Leu4 to Phe29 of 6G4V11N35A) (Fig. 47) and a 291bp XhoI - ApaI fragment from p6G4V11N35A.7 carrying the remainder of the variable heavy chain sequence of 6G4V11N35A to create pSL-3. This intermediate plasmid was used in conjunction with 2 other plasmids, p6G4V11N35A.F(ab')<sub>2</sub> and p6G425chim2.choSD, to create the mammalian expression plasmid, p6G4V11N35AchoSD.9 (identified as p6G425V11N35A.choSD in a deposit made on December 16, 1997 with the ATCC and assigned ATCC Accession No. 209552). This expression construct was assembled in a 4-part ligation using the following DNA fragments: a 5,203bp ClaI - BlnI fragment encoding the regulatory elements of the mammalian expression plasmid (p6G425 chim2.choSD), a 451bp ClaI - ApaI fragment containing the heavy chain variable region of the

humanized 6G4V11N35A antibody (pSL-3), a 1,921bp *ApaI* - *EcoRV* fragment carrying the heavy chain constant region of 6G4V11N35A (p6G425chim2.choSD) and a 554bp *EcoRV* - *BlpI* fragment encoding the light chain variable and constant regions of 6G4V11N35A (p6G4V11N35A.F(ab')<sub>2</sub>). The DNA sequence (SEQ ID NO: 68) of clone p6G4V11N35A.choSD.9 was confirmed by DNA sequencing and is presented in Figure 48.

Q. CONSTRUCTION OF THE FULL LENGTH HUMANIZED 6G4V11N35E IgG EXPRESSION PLASMID

A mammalian expression vector for the humanized 6G4V11N35E was made by swapping the light chain variable region of 6G4V11N35A with 6G4V11N35E as follows: a 7,566bp *EcoRV* - *BlpI* fragment (void of the 554bp fragment encoding the light chain variable region of 6G4V11N35A) from p6G4V11N35A.choSD.9 was ligated to a 554bp *EcoRV* - *BlpI* fragment (encoding the light chain variable region of 6G4V11N35E) from pPH6G4V11N35E.7. The mutation at position N35 of the light chain of p6G4V11N35E.choSD.10 was confirmed by DNA sequencing.

R. STABLE CHO CELL LINES FOR VARIANTS N35A AND N35E

For stable expression of the final humanized IgG1 variants (6G4V11N35A and 6G4V11N35E), Chinese hamster ovary (CHO) DP-12 cells were transfected with the above-described dicistronic vectors (p6G4V11N35A.choSD.9 and p6G4V11N35E.choSD.10, respectively) designed to coexpress both heavy and light chains (Lucas et al., *Nucleic Acid Res.* 24:1774-79 (1996)). Plasmids were introduced into CHO DP12 cells via lipofection and selected for growth in GHT-free medium (Chisholm, V. High efficiency gene transfer in mammalian cells. In: Glover, DM, Hames, BD. *DNA Cloning 4. Mammalian systems*. Oxford Univ. Press, Oxford pp 1-41 (1996)). Approximately 20 unamplified clones were randomly chosen and reseeded into 96 well plates. Relative specific productivity of each colony was monitored using an ELISA to quantitate the full length human IgG accumulated in each well after 3 days and a fluorescent dye, Calcein AM, as a surrogate marker of viable cell number per well. Based on these data, several unamplified clones were chosen for further amplification in the presence of increasing concentrations of methotrexate. Individual clones surviving at 10, 50, and 100 nM methotrexate were

chosen and transferred to 96 well plates for productivity screening. One clone for each antibody (clone#1933 aIL8.92 NB 28605/12 for 6G4V11N35A; clone#1934 aIL8.42 NB 28605/14 for 6G4V11N35E), which reproducibly exhibited high specific productivity, was expanded in T-flasks and used to inoculate a spinner culture. After several passages, the suspension-adapted cells were used to inoculate production cultures in GHT-containing, serum-free media supplemented with various hormones and protein hydrolysates. Harvested cell culture fluid containing recombinant humanized anti-IL8 was purified using protein A-Sepharose CL-4B. The purity after this step was approximately 99% . Subsequent purification to homogeneity was carried out using an ion exchange chromatography step. Production titer of the humanized 6G4V11N35E IgG1 antibody after the first round of amplification and 6G4V11N35A IgG1 after the second round of amplification were 250mg/L and 150mg/L, respectively.

#### S. CHARACTERIZATION OF THE HUMANIZED 6G4V11N35A/E IgG VARIANTS

The humanized full length IgG variants of 6G4.2.5 were tested for their ability to inhibit <sup>125</sup>I-IL-8 binding and to neutralize activation of human neutrophils; the procedures are described in Sections (B)(1) and (B)(2) above. As shown in Figure 49, the full length IgG1 forms of variants 6G4V11N35A and 6G4V11N35E equally inhibited <sup>125</sup>I-IL-8 binding to human neutrophils with approximate IC<sub>50</sub>'s of 0.3nM and 0.5nM, respectively. This represents a 15 - 25 fold improvement in blocking binding of IL-8 compared to the full length murine mAb (IC<sub>50</sub> = 7.5nM). Similarly, the two anti-IL-8 variants showed equivalent neutralizing capabilities with respect to inhibiting IL-8 mediated human neutrophil chemotaxis (Figures 50A-50B). The IC<sub>50</sub>'s of 6G4V11N35A IgG1 and 6G4V11N35E IgG1 for human IL-8 were 4.0nM and 6.0nM, respectively, and for rabbit IL-8 were 4.0nM and 2.0nM, respectively. The irrelevant isotype control Fab (4D5) did not inhibit neutrophil migration.

The affinity for IL-8 of these variants relative to the murine 6G4.2.5 mAb was determined using KinExA as described in Section (M). Figure 51 shows the equilibrium constant (Kd) for the full length affinity matured variants 6G4V11N35E IgG1 and 6G4V11N35A IgG1 were approximately 49pM and 88pM, respectively. The Kd for 6G4V11N35A IgG1 was determined directly from the kinetic experiment. As reported with their respective Fabs, this improvement in affinity might be attributed to an approximate 2-fold increase in the on-rate of 6G4V11N35E IgG1 ( $k_a = 3.0 \times 10^6$ ) compared to that of

6G4V11N35A IgG1 ( $k_a = 8.7 \times 10^5$ ). In addition, these results were confirmed by a competition radio-immune assay using iodinated human IL-8. 50pM of 6G4V11N35A IgG1 or 6G4V11N35E IgG1 was incubated for 2 hours at 25°C with 30-50pM of  $^{125}$ I-IL-8 and varying concentrations (0 to 100nM) of unlabeled IL-8. The antibody-antigen mixture was then incubated for 1 hour at 4°C with 10ul of a 70% slurry of Protein-A beads (pre-blocked with 0.1% BSA). The beads were briefly spun in a microcentrifuge and the supernatant discarded to remove the unbound  $^{125}$ I-IL-8. The amount of  $^{125}$ I-IL-8 specifically bound to the anti-IL-8 antibodies was determined by counting the protein-A pellets in a gamma counter. The approximate Kd values were similar to those determined by KinEXA. The average Kd for 6G4V11N35A IgG1 and 6G4V11N35E IgG1 were 54pM (18 -90pM) and 19pM (5-34pM), respectively (Figure 52).

#### T. CONSTRUCTION OF HUMANIZED 6G4V11N35A/E Fab's FOR MODIFICATION BY POLYETHYLENE GLYCOL

A Fab' expression vector for 6G4V11N35A was constructed by digesting p6G4V11N35A.F(ab')<sub>2</sub> with the restriction enzymes ApaI and NdeI to remove the 2805 bp fragment encoding the human IgG<sub>1</sub> constant domain fused with the yeast GCN4 leucine zipper and replacing it with the 2683bp ApaI-NdeI fragment from the plasmid pCDNA.18 described in Eigenbrot et al., Proteins: Struct. Funct. Genet., **18**: 49-62 (1994). The pCDNA.18 ApaI-NdeI fragment carries the coding sequence for the human constant IgG1 heavy domain, including the free cysteine in the hinge region that was used to attach the PEG molecule. The 3758bp ApaI-NdeI fragment (encodes the light chain and heavy variable domain of 6G4V11N35A) isolated from p6G4V11N35A.F(ab')<sub>2</sub> was ligated to the 2683bp ApaI-NdeI fragment of pCDNA.18 to create p6G4V11N35A.PEG-1. The integrity of the entire coding sequence was confirmed by DNA sequencing. The nucleotide and translated amino acid sequences of heavy chain constant domain with the cysteine in the hinge are presented in Figure 53.

A Fab' expression plasmid for 6G4V11N35E was made similarly by digesting pPH6G4V11N35E (from Section (O) above) with the restriction enzymes ApaI and NdeI to isolate the 3758bp ApaI-NdeI DNA fragment carrying the intact light chain and heavy variable domain of 6G4V11N35E and ligating it to the 2683 bp ApaI-NdeI DNA fragment from p6G4V11N35A.PEG-1 to

create p6G4V11N35E.PEG-3. The integrity of the entire coding sequence was confirmed by DNA sequencing.

Anti-IL-8 6G4V11N35A Fab' variant was modified with 20 kD linear methoxy-PEG-maleimide, 30 kD linear methoxy-PEG-maleimide, 40 kD linear methoxy-PEG-maleimide, or 40 kD branched methoxy-PEG-maleimide as described below. All PEG's used were obtained commercially from Shearwater Polymers, Inc.

#### a. MATERIALS AND METHODS

##### Fab'-SH Purification

A Fab'-SH antibody fragment of the affinity matured antibody 6G4V11N35A was expressed in *E. coli* grown to high cell density in the fermentor as described by Carter *et al.*, *Bio/Technology* **10**, 163–167 (1992). Preparation of Fab'-SH fragments was accomplished by protecting the Fab'-SH fragments with 4',4'-dithiodipyridine (PDS), partially purifying the protected Fab'-PDS fragments, deprotect the Fab'-PDS with dithiothreitol (DTT) and finally isolate the free Fab'-SH by using gel permeation chromatography.

##### Protection of Fab'-SH with PDS

Fermentation paste samples were dissolved in 3 volumes of 20mM MES, 5mM EDTA, pH 6.0 containing 10.7mg of 4',4'-dithiodipyridine per gram fermentation paste, resulting in a suspension with a pH close to 6.0 The suspension was passed through a homogenizer followed by addition of 5% PEI (w/v), pH 6 to the homogenate to a final concentration of 0.25%. The mixture was then centrifuged to remove solids and the clear supernatant was conditioned to a conductivity of less than 3mS by the addition of cold water.

Partial purification of the Fab'-SH molecule using ion exchange chromatography

The conditioned supernatant was loaded onto an ABX (Baker) column equilibrated in 20 mM MES, pH 6.0. The column was washed with the equilibration buffer followed by elution of the Fab'-SH with a 15 column volume linear gradient from 20 mM MES, pH 6.0 to 20 mM MES, 350 mM sodium chloride. The column was monitored by absorbance at 280nm, and the eluate was collected in fractions.

Deprotection of the Fab'-SH antibody fragments with DTT

The pH of the ABX pool was adjusted to 4.0 by the addition of dilute HCl. The pH adjusted solution was then deprotected by adding DTT to a final concentration of 0.2mM. The solution was incubated for about 30 minutes and then applied to a gel filtration Sephadex G25 column, equilibrated with 15mM sodium phosphate, 25mM MES, pH 4.0. After elution, the pH of the pool was raised to pH 5.5 and immediately flash frozen at -70°C for storage or derivatized with PEG-MAL as described below.

Alternative Fab'-SH Purification

Alternatively Fab'-SH fragments can be purified using the following procedure. 100 g fermentation paste is thawed in the presence of 200 ml 50 mM acetic acid, pH 2.8, 2 mM EDTA, 1 mM PMSF. After mixing vigorously for 30 min at room temperature, the extract is incubated with 100 mg hen egg white lysozyme. DEAE fast flow resin (approximately 100 mL) is equilibrated with 10 mM MES, pH 5.5, 1 mM EDTA on a sintered glass funnel. The osmotic shock extract containing the Fab'-SH fragment is then filtered through the resin.

A protein G Sepharose column is equilibrated with 10 mM MES, pH 5.5, 1 mM EDTA and then loaded with the DEAE flow-through sample. The column is washed followed by three 4 column volume washes with 10 mM MES, pH 5.5, 1 mM EDTA. The Fab'-SH antibody

fragment containing a free thiol is eluted from the column with 100 mM acetic acid, pH 2.8, 1 mM EDTA. After elution, the pH of the pool is raised to pH 5.5 and immediately flash frozen at  $-70^{\circ}\text{C}$  for storage or derivatized with PEG-MAL as described below.

#### Preparation of Fab'-S-PEG

5 The free thiol content of the Fab'-SH preparation obtained as described above was determined by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) analysis according to the method of Creighton in Protein Structure: A Practical Approach, Creighton, T.E., ed, IRL Press (Oxford, UK: 1990), pp. 155-167. The concentration of free thiol was calculated from the increase on absorbance at 412 nm, using  $\epsilon_{412} = 14,150 \text{ cm}^{-1} \text{ M}^{-1}$  for the thionitrobenzoate anion and a  $M_r = 48,690$  and  $\epsilon_{280} = 1.5$  for the Fab'-SH antibody. To the Fab'-SH protein G Sepharose pool, or the deprotected Fab'-SH gel permeation pool, 5 molar equivalents of PEG-MAL were added and the pH was immediately adjusted to pH 6.5 with 10% NaOH.

10 The Fab'-S-PEG was purified using a 2.5 x 20 cm cation exchange column (Poros 50-HS). The column was equilibrated with a buffer containing 20 mM MES, pH 5.5. The coupling reaction containing the PEGylated antibody fragment was diluted with deionized water to a conductivity of approximately 2.0 mS. The conditioned coupling reaction was then loaded onto the equilibrated Poros 50 HS column. Unreacted PEG-MAL was washed from the column with 2 column volumes of 20 mM MES, pH 5.5. The Fab'-S-PEG was eluted from the column using a linear gradient from 0 to 400 mM NaCl, in 20 mM MES pH 5.5, over 15 column volumes.

20 Alternatively a Bakerbond ABX column can be used to purify the Fab'-S-PEG molecule. The column is equilibrated with 20 mM MES, pH 6.0 (Buffer A). The coupling reaction is diluted with deionized water until the conductivity equaled that of the Buffer A (approximately 2.0 mS) and loaded onto the column. Unreacted PEG-MAL is washed from the column with 2 column volumes of 20 mM MES, pH 6.0. The Fab'-S-PEG is eluted from the

column using a linear gradient from 0 to 100 mM  $(\text{NH}_4)_2\text{SO}_4$ , in 20 mM MES pH 6.0, over 15 column volumes.

#### Size Exclusion Chromatography

The hydrodynamic or effective size of each molecule was determined using a Pharmacia Superose-6 HR 10/30 column (10x300mm) . The mobile phase was 200 mM NaCl, 50 mM sodium phosphate pH 6.0. Flow rate was at 0.5 ml/min and the column was kept at ambient temperature. Absorbance at 280 nm was monitored where PEG contributed little signal. Biorad MW standards containing cyanocobalamin, myoglobin, ovalbumin, IgG, Thyroglobulin monomer and dimer were used to generate a standard curve from which the effective size of the pegylated species was estimated.

### b. RESULTS

#### Size Exclusion Chromatography

The effective size of each modified species was characterized using size exclusion chromatography. The results are shown in Fig. 60 below. The theoretical molecular weight of the anti-IL8 Fab fragments modified with PEG 5kD, 10kD, 20kD, 30kD, 40kD (linear), 40kD (branched) or 100,000kD is shown along with the apparent molecular weight of the PEGylated fragments obtained by HPLC size exclusion chromatography. When compared to the theoretical molecular weight of the Fab'-S-PEG fragments, the apparent molecular weight (calculated by size exclusion HPLC) increases dramatically by increasing the size of the PEG attached to the fragments. Attachment of a small molecular weight PEG, for example PEG 10,000D only increases the theoretical molecular weight of the PEGylated antibody fragment (59,700 D) by 3 fold to an apparent molecular weight of 180,000D. In contrast attachment of a larger molecular weight PEG for example 100,000D PEG to the antibody fragment increases the theoretical molecular weight of the PEGylated antibody fragment (158,700 D) by 12 fold to an apparent molecular weight of 2,000,000D.



SDS-PAGE

In Fig. 61, the upper panel shows the size of the anti-IL-8 Fab fragments modified with PEG of molecular weight 5kD (linear), 10kD (linear), 20kD (linear), 30kD (linear), 40kD (linear), 40kD (branched) or 100kD (linear) under reduced conditions. The unmodified Fab is shown in lane 2 from right to left. Both the heavy and light chains of the Fab had a molecular weight of approximately 30kD as determined by PAGE. Each PEGylated fragment sample produced two bands: (1) a first band (attributed to the light chain) exhibiting a molecular weight of 30kD; and (2) a second band (attributed to the heavy chain to which the PEG is attached specifically at the hinge SH) exhibiting increasing molecular weights of 40, 45, 70, 110, 125, 150 and 300kD. This result suggested that PEGylation was specifically restricted to the heavy chain of the Fab's whereas the light chain remained unmodified.

The lower panel is non-reduced PAGE showing the size of the anti-IL-8 Fab fragments modified with PEG of molecular weight 5kD (linear), 20kD (linear), 30kD (linear), 40kD (linear), 40kD (branched), or 100kD (linear). The PEGylated fragments exhibited molecular weights of approximately 70kD, 115kD, 120kD, 140kD, 200kD and 300kD.

The SDS PAGE gels confirm that all Fab'-S-PEG molecules were purified to homogeneity and that the molecules differed only with respect to the size of the PEG molecule attached to them.

#### U. AMINE SPECIFIC PEGYLATION OF ANTI-IL-8 F(ab')<sub>2</sub> FRAGMENTS

Pegylated F(ab')<sub>2</sub> species were generated by using large MW or branched PEGs in order to achieve a large effective size with minimal protein modification which might affect activity. Modification involved N-hydroxysuccinamide chemistry which reacts with primary amines (lysines and the N-terminus). To decrease the probability of modifying the N-terminus, which is in close proximity to the CDR region, a reaction pH of 8, rather than the commonly used pH of 7, was employed. At pH 8.0, the amount of the reactive species (charged NH<sub>3</sub><sup>+</sup>) would be considerably more for the ε-NH<sub>2</sub> group of lysines (pK<sub>a</sub>=10.3) than for the α-NH<sub>2</sub> group (pK<sub>a</sub> of

approximately 7) of the amino-terminus. For the linear PEGs, a methoxy-succinimidyl derivative of an NHS-PEG was used because of the significantly longer half-life in solution (17 minutes at 25°C at pH 8.0) compared to the NHS esters of PEGs (which have 5-7 minute half life under the above conditions). By using a PEG that is less prone to hydrolysis, a greater extent of modification is achieved with less PEG. Branched PEGs were used to induce a large increase in effective size of the antibody fragments.

#### a. MATERIALS

All PEG reagents were purchased from Shearwater Polymers and stored at -70°C in a desiccator: branched N-hydroxysuccinamide-PEG (PEG2-NHS-40KDa) has a 20 kDa PEG on each of the two branches, methoxy-succinimidyl-propionic acid-PEG (M-SPA-20000) is a linear PEG molecule with 20 kDa PEG. Protein was recombinantly produced in *E. coli* and purified as a (Fab)<sub>2</sub> as described in Sections (K) and (O) above.

#### b. METHODS

**IEX method:** A J. T. Baker Wide-Pore Carboxy-sulfone (CSX), 5 micron, 7.75 x 100 mm HPLC column was used for fractionation of the different pegylated products, taking advantage of the difference in charge as the lysines are modified. The column was heated at 40°C. A gradient as shown in Table 7 below was used where Buffer A was 25 mM sodium Borate/25 mM sodium phosphate pH 6.0, and Buffer B was 1 M ammonium sulfate, and Buffer C was 50 mM sodium acetate pH 5.0.

**Table 7**

<i>Time (min)</i>	<i>%B</i>	<i>%C</i>	<i>flow mL/min</i>
0	10	10	1.5
20	18	7.5	1.5
25	25	7.5	1.5
27	70	3.0	2.5
29	70	3.0	2.5
30	10	10	2.5
33	10	10	2.5

**SEC-HPLC:** The hydrodynamic or effective size of each molecule was determined using a Pharmacia Superose-6 HR 10/30 column (10x300mm) . The mobile phase was 200 mM NaCl, 50 mM sodium phosphate pH 6.0. Flow rate was at 0.5 ml/min and the column was kept at ambient temperature. Absorbance at 280 nm was monitored where PEG contributed little signal. Biorad MW standards containing cyanocobalamin, myoglobin, ovalbumin, IgG, Thyroglobulin monomer and dimer were used to generate a standard curve from which the effective size of the pegylated species was estimated.

**SEC-HPLC-Light Scattering:** For determination of the exact molecular weight, this column was connected to an on-line light scattering detector (Wyatt Minidawn) equipped with three detection angles of 50°, 90°, and 135° C. A refractive index detector (Wyatt) was also placed on-line to determine concentration. All buffers were filtered with Millipore 0.1 µ filters; in addition al 0.02 µ Whatman Anodisc 47 was placed on-line prior to the column.

The intensity of scattered light is directly proportional to the molecular weight (M) of the scattering species, independent of shape, according to:

$$M = R_0 / K \cdot c$$

where  $R_0$  is the Rayleigh ratio,  $K$  is an optical constant relating to the refractive index of the solvent, the wavelength of the incident light, and  $dn/dc$ , the differential refractive index between the solvent and the solute with respect to the change in solute concentration,  $c$ . The system was

calibrated with toluene ( $R_0$  of  $1.406 \times 10^{-5}$  at 632.8 nm); a  $dn/dc$  of 0.18, and an extinction coefficient of 1.2 was used. The system had a mass accuracy of ~5%.

**SDS-PAGE:** 4-12% Tris-Glycine Novex minigels were used along with the Novex supplied Tris-Glycine running buffers. 10-20 ug of protein was applied in each well and the gels were run in a cold box at 150 mV/gel for 45 minutes. Gels were then stained with colloidal Coomassie Blue (Novex) and then washed with water for a few hours and then preserved and dried in drying buffer (Novex)

**Preparation of a linear(1)20KDa-(N)-(Fab')<sub>2</sub>:** A 4 mg/ml solution of anti-IL8 formulated initially in a pH 5.5 buffer was dialyzed overnight against a pH 8.0 sodium phosphate buffer. 5 mL protein was mixed at a molar ratio of 3:1. The reaction was carried out in a 15mL polypropylene Falcon tube and the PEG was added while vortexing the sample at low speed for 5 seconds. It was then placed on a nutator for 30 minutes. The extent of modification was evaluated by SDS-PAGE. The whole 5 ml reaction mixture was injected on the IEX for removal of any unreacted PEG and purification of singly or doubly pegylated species. The above reaction generated a mixture of 50% singly-labeled anti-IL8. The other 50% unreacted anti-IL8 was recycled through the pegylation/purification steps. The pooled pegylated product was dialyzed against a pH 5.5 buffer for in vitro assays and animal PK studies. Endotoxin levels were measured before administration to animals or for the cell based assays. Levels were below 0.5 eu/ml. The fractions were also run on SDS-PAGE to confirm homogeneity. Concentration of the final product was assessed by absorbance at 280 nm using an extinction coefficient of 1.34, as well as by amino acid analysis.

**Preparation of a branched(1)40KDa-(N)-(Fab')<sub>2</sub>:** A 4 mg/mL solution of anti-IL8 (Fab')<sub>2</sub> formulated in a pH 5.5 buffer was dialyzed overnight against a pH 8.0 phosphate buffer. Solid PEG powder was added to 5 mL protein in two aliquots to give a final PEG:protein molar ratio of 6:1. Each solid PEG aliquot was added to the protein in a 15 mL polypropylene Falcon tube while vortexing at low speed for 5 sec, and then placing the sample on a nutator for 15 minutes. The extent of modification was evaluated by SDS-PAGE using a 4-12% Tris-Glycine (Novex) gel and stained with colloidal Coomassie blue (Novex). The 5 mL PEG-protein mixture

was injected on the ion exchange column for removal of any unreacted PEG. The above reaction generated a mixture of unreacted (37%), singly-labelled (45%), doubly and triply-labeled (18%) species. These were the optimal conditions for obtaining the greatest recovery of the protein with only 1 PEG per antibody rather than the higher molecular weight adducts. The unmodified anti-IL8 was recycled. The pegylated products were separated and fractionated in falcon tubes and then dialyzed against a pH 5.5 buffer for assays and animal PK studies. Endotoxin levels were below 0.5 eu/ml. The fractions were also run on SDS-PAGE to confirm homogeneity. The concentration of the final product was assessed by absorbance at 280 nm using an extinction coefficient of 1.34, as well as by amino acid analysis.

**Preparation of branched(2)-40KDa-(N)-(Fab')<sub>2</sub>:** This molecule was most efficiently made by adding three times in 15 minute intervals a 3:1 molar ratio of PEG to the already modified branched(1)-40KDa-(N)-(Fab')<sub>2</sub>. The molecule was purified on IEX as 50% branched(2)-40KDa-(N)-(Fab')<sub>2</sub>. The unmodified molecule was recycled until ~20 mg protein was isolated for animal PK studies. The product was characterized by SEC-light scattering and SDS-PAGE.

### c. RESULTS

PEGs increased the hydrodynamic or effective size of the product significantly as determined by gel filtration (SEC-HPLC). Figure 62 shows the SEC profile of the pegylated F(ab')<sub>2</sub> species with UV detection at 280 nm. The hydrodynamic size of each molecule was estimated by reference to the standard MW calibrators. As summarized in Figure 62, the increase in the effective size of (Fab')<sub>2</sub> was about 7-fold by adding one linear 20 kDa PEG molecule and about 11-fold by adding one branched ("Br(1)") 40 kDa PEG molecule, and somewhat more with addition of two branched ("Br(2)") PEG molecules.

Light scattering detection gave the exact molecular weight of the products and confirmed the extent of modification (Figure 63). The homogeneity of the purified material was shown by SDS-PAGE (Figure 64). Underivatized F(ab')<sub>2</sub> migrated as a 120 kDa species, the linear(1)20KD-(N)-F(ab')<sub>2</sub> migrated as a band at 220kDa, the Br(1)-40KD(N)-F(ab')<sub>2</sub> migrated

as one major band at 400 kDa, and the Br(2)-40KD-(N)-F(ab')<sub>2</sub> migrated as a major band at around 500 kDa. The proteins appeared somewhat larger than their absolute MW due to the steric effect of PEG.

5 V. IN VITRO ACTIVITY CHARACTERIZATION OF PEG MODIFIED Fab' FRAGMENTS OF 6G4V11N35A (MALEIMIDE CHEMICAL COUPLING METHOD)

Anti-IL-8 6G4V11N35A Fab' variants modified with 5-40kD linear PEG molecules and a 40kD branched PEG molecule were tested for their ability to inhibit both IL-8 binding and activation of human neutrophils; the procedures were described in Sections (B)(1), (B)(2) and (B)(3) above. The binding  
 10 curves and IC<sub>50</sub>'s for PEG-maleimide modified 6G4V11N35A Fab' molecules are presented in Figures 54A-54C. The IC<sub>50</sub> of the 5kD pegylated Fab' (350pM) and the average IC<sub>50</sub> of the Fab control (366pM) were not significantly different, suggesting that the addition of a 5kD MW PEG did not affect the binding of IL-8 to the modified Fab' (Figure 54A). However, a decrease in the binding of IL-8 to the 10kD and 20kD pegylated Fab' molecules was observed as depicted by the progressively higher  
 15 IC<sub>50</sub>'s (537pM and 732pM, respectively) compared to the average IC<sub>50</sub> of the native Fab. These values represent only a minimal loss of binding activity (between 1.5- and 2.0-fold). A less pronounced difference in IL-8 binding was observed for the 30kD and 40kD linear PEG antibodies (Figure 54B). The IC<sub>50</sub>'s were 624pM and 1.1nM, respectively, compared to the 802pM value of the Fab control. The 40kD branched PEG Fab' showed the largest decrease in IL-8 binding (2.5 fold) relative to the native Fab (Figure 54C). Nevertheless, the reduction in binding of IL-8 by these pegylated Fab's is minimal.  
 20

The ability of the pegylated antibodies to block IL-8 mediated activation of human neutrophils was demonstrated using the PMN chemotaxis (according to the method described in Section B(2) above) and β-glucuronidase release (according to the method described in Lowman et al., *J. Biol. Chem.*, 271: 14344 (1996)) assays. The IC<sub>50</sub>'s for blocking IL-8 mediated chemotaxis are shown in Figures 55A-  
 25 55C. The 5-20kD linear pegylated Fab' antibodies were able to block IL-8 mediated chemotaxis within 2-3 fold of the unpegylated Fab control (Figure 55A). This difference is not significant because the inherent variation can be up to 2 fold for this type of assay. However, a significant difference was detected for the 30kD and 40kD linear pegylated Fab' antibodies as illustrated by the higher IC<sub>50</sub>'s of

the 30kD linear PEG-Fab' (2.5nM) and 40kD linear PEG-Fab' (3.7nM) compared to the Fab control (0.8nM) (Figure 55B). The ability of the 40kD branched PEG Fab' molecule to block IL-8 mediated chemotaxis was similar to that of the 40kD linear PEG Fab' (Figure 55C). At most, the ability of the pegylated Fab' antibodies to block IL-8 mediated chemotaxis was only reduced 2-3 fold. Furthermore, release of  $\beta$ -glucuronidase from the granules of neutrophils was used as another criteria for assessing IL-8 mediated activation of human PMNs. Figure 56A (depicting results obtained with 5 kD, 10 kD and 20 kD linear PEGs), Figure 56B (depicting results obtained with 30 kD and 40 kD linear PEGs), and Figure 56C (depicting results obtained with 40 kD branched PEG) show that all the pegylated Fab' antibodies were able to inhibit IL-8 mediated release of  $\beta$ -glucuronidase as well as or better than the unpegylated Fab control. The data collectively shows that the pegylated Fab' variants are biological active and are capable of inhibiting high amounts of exogenous IL-8 in in-vitro assays using human neutrophils.

#### W. IN VITRO ACTIVITY CHARACTERIZATION OF PEG MODIFIED F(ab')<sub>2</sub> FRAGMENTS OF 6G4V11N35A (SUCCINIMIDYL CHEMICAL COUPLING METHOD)

The anti-IL-8 variant 6G4V11N35A F(ab')<sub>2</sub> modified with (a) a single 20kD linear PEG molecule per F(ab')<sub>2</sub>, (b) a single 40kD branched PEG molecule per F(ab')<sub>2</sub>, (c) with three, four, or five 20 kD linear PEG molecules per F(ab')<sub>2</sub> (a mixture of: (1) species having three 20 kD linear PEG molecules per F(ab')<sub>2</sub>; (2) species having four 20 kD linear PEG molecules per F(ab')<sub>2</sub>; and (3) species having five 20 kD linear PEG molecules per F(ab')<sub>2</sub>; denoted as "20 kD linear PEG (3,4,5) F(ab')<sub>2</sub>"), or (d) with two 40kD branched PEG molecules per F(ab')<sub>2</sub> (denoted as "40 kD branch PEG (2) F(ab')<sub>2</sub>"), were tested for their ability to inhibit <sup>125</sup>I-IL-8 binding and to neutralize activation of human neutrophils. The procedures used are described in Sections (B)(1), (B)(2) and (B)(3) above. The binding curves for pegylated F(ab')<sub>2</sub> variants are shown in Figures 57A-57B. No significant differences were observed amongst the F(ab')<sub>2</sub> control, the single 20kD linear PEG-modified F(ab')<sub>2</sub>, and the single 40kD branched PEG-modified F(ab')<sub>2</sub> (Figure 57A). However, the F(ab')<sub>2</sub> variants containing multiple PEG molecules showed a slight reduction (less than 2-fold) in their ability to bind IL-8. The IC<sub>50</sub>'s of the 20kD linear PEG (3,4,5) F(ab')<sub>2</sub> and 40kD branch PEG (2) F(ab')<sub>2</sub> variants were 437pM and 510pM, respectively, compared to 349pM of the F(ab')<sub>2</sub> control (Figure 57B).

The ability of these pegylated  $F(ab')_2$  variants to block IL-8 mediated neutrophil chemotaxis is presented in Figures 58A-58B. Consistent with the PMN binding data, the single linear and branched PEG  $F(ab')_2$  variants were able to block IL-8 mediated chemotaxis similar to the unpegylated  $F(ab')_2$  control (Figure 58A). The ability of the 40kD branch PEG (2)  $F(ab')_2$  variant to inhibit PMN chemotaxis was identical to the control  $F(ab')_2$  while the 20kD linear PEG (3,4,5)  $F(ab')_2$  mixture was able to inhibit within 3-fold of the control antibody (Figure 58B).

Shown in Figures 59A and 59B are the results of the  $\beta$ -glucuronidase release assay which is a measure of degranulation by IL-8 stimulated human neutrophils. The single 20kD linear PEG-modified  $F(ab')_2$  and the single 40kD branched PEG-modified  $F(ab')_2$  variants were able to inhibit release of  $\beta$ -glucuronidase as well as the  $F(ab')_2$  control (Figure 59A). The 40kD branch PEG (2)  $F(ab')_2$  inhibited this response within 2-fold of the  $F(ab')_2$  control (Figure 59B). The 20kD linear PEG (3,4,5) molecule was not tested. Overall, the  $F(ab')_2$  pegylated anti-IL-8 antibodies were biologically active and effectively prevented IL-8 binding to human neutrophils and the signaling events leading to cellular activation.

#### X. PHARMACOKINETIC AND SAFETY STUDY OF EIGHT CONSTRUCTS OF PEGYLATED ANTI-IL-8 (HUMANIZED) $F(ab')_2$ AND FAB' FRAGMENTS IN NORMAL RABBITS FOLLOWING INTRAVENOUS ADMINISTRATION

The objective of this study was to evaluate the effect of pegylation on the pharmacokinetics and safety of six pegylated humanized anti-IL-8 constructs (pegylated 6G4V11N35A.Fab' and pegylated 6G4V11N35A. $F(ab')_2$  obtained as described in Sections (T) and (U) above) relative to the non-pegylated fragments in normal rabbits. Eight groups of two/three male rabbits received equivalent protein amounts of pegylated 6G4V11N35A.Fab' or pegylated 6G4V11N35A. $F(ab')_2$  constructs (2 mg/kg) via a single intravenous (IV) bolus dose of one anti-IL8 construct. Serum samples were collected according to the schedule shown in Table 8 below and analyzed for anti-IL8 protein concentrations and antibody formation against anti-IL8 constructs by ELISA.



**Table 8**

Group No.	Dose level/ Route	Material	Blood Collection
1	2 mg/kg (protein conc.) IV bolus	Fab' control	0,5,30 min; 1,2,3,4,6,8,10, 14,20,24,360 hr
2		linear(1)20K(s)Fab'	0,5,30 min; 1,2,4,6,8,10,12, 24,28,32,48,72,96,168,216, 264,336,360 hr
3		linear(1)40K(s)Fab'	
4		branched(1)40K(N)F(ab') <sub>2</sub>	
5		F(ab') <sub>2</sub> control	0,5,30 min; 1,2,4,6,8,10,12, 24,28,32,48,52,56,336 hr
6		branched(2)40K(s)Fab'	0,5,30 min; 1,2,4,6,8,10,12, 24,28,32,48,72,96,168,216,26 4,336 hr; Day 17,21, 25
7		branched(2)40K(N)F(ab') <sub>2</sub>	0,5,30 min; 1,2,4,6,8,10,12, 24,28,32,48,72,144,192, 240 hr; Day 13, 16, 20, 23
8		linear(1)30K(s)Fab'	0,5,30 min; 1,2,4,6,8,10,12, 24,28,32,48,72,96,168,216,26 4,336 hr; Day 17,21, 25

a. METHODS

- 5 Three male New Zealand White (NZW) rabbits per group (with exception to Group 7, n=2) received an equivalent amount of 6G4V11N35A protein (Fab' or F(ab')<sub>2</sub>) construct at 2 mg/kg via an IV bolus dose in a marginal ear vein. Amino acid composition analysis and absorbance at 280 nm using extinction coefficients of 1.26 for 6G4V11N35A Fab' constructs and 1.34 for 6G4V11N35A F(ab')<sub>2</sub> constructs were performed to determine the protein concentration.
- 10 Whole blood samples were collected via an ear artery cannulation (ear opposing dosing ear) at the above time points. Samples were harvested for serum and assayed for free 6G4V11N35A

Fab' or F(ab')<sub>2</sub> constructs using an IL-8 Binding ELISA. Assays were conducted throughout the study as samples became available. All animals were sacrificed following the last blood draw, and necropsies were performed on all animals in Groups 1, 4–8. Due to the development of antibodies against the 6G4V11N35A constructs, non-compartmental pharmacokinetic analysis was conducted on concentration versus time data only up to 168 hours.

#### b. RESULTS

In four animals (Animals B, P, Q, V), interference to rabbit serum in the ELISA assay was detected (i.e. measurable concentrations of anti-IL8 antibodies at pre-dose). However, because these values were at insignificant levels and did not effect the pharmacokinetic analysis, the data were not corrected for this interference.

One animal (Animal G; Group 3) was exsanguinated before the termination of the study and was excluded from the pharmacokinetic analysis. At 4 hours, the animal showed signs of a stroke that was not believed to be drug related, as this can occur in rabbits following blood draws via ear artery cannulation.

The mean concentration–time profiles of the eight anti-IL8 constructs in normal rabbits are depicted in Fig. 65, and the pharmacokinetic parameters for the eight constructs are summarized in Table 9 below. Significant antibodies to the anti-IL-8 constructs were present at Day 13/14 in all dose groups except Group 1 (Fab' control).

**Table 9. Pharmacokinetic parameters.**

Molecule	Fab'					F(ab') <sub>2</sub>		
Group No.	1	2	8	3	6	5	4	7
PEG structure	—	linear	linear	linear	branched	—	branched	branched
Number of PEGs	—	1	1	1	1	—	1	2
PEG MW	—	20K	30K	40K	40K	—	40K	40K
Dose (mg/kg)	2	2	2	2	2	2	2	2
V <sub>c</sub> (mL/kg) <sup>a</sup>	58±3	36±3	35±1	34	44±1	45±5	36±1	32
V <sub>ss</sub> (mL/kg) <sup>b</sup>	68±8	80±8	110±15	79	88±21	59±4	50±3	52
C <sub>max</sub> (µg/mL) <sup>c</sup>	35±1	58±3	57±1	60	45±1	45±6	56±2	62
T <sub>max</sub> (min) <sup>d</sup>	5	5	5	5	5	5	5	5
t <sub>1/2</sub> term (hr) <sup>e</sup>	3.0±0.9	44±2	43±7	50	105±11	8.5±2.1	45±3	48
AUC <sub>0-8</sub> (hr•µg/mL) <sup>f</sup>	18±3	80±74	910±140	1600	3400±1300	140±3	2200±77	2500
CL (mL/hr/kg) <sup>g</sup>	110±17	2.5±0.2	2.2±0.4	1.3	0.63±0.20	14±0	0.92±0.03	0.83
MRT (hr) <sup>h</sup>	0.61±0.15	32±2	45±9	63	140±18	4.2±0.3	55±3	64
No. of Animals	3	3	3	2	3	3	3	2

<sup>a</sup> Initial volume of distribution.<sup>b</sup> Volume of distribution at steady state.<sup>c</sup> Observed maximum concentration.<sup>d</sup> Observed time to C<sub>max</sub>.<sup>e</sup> t<sub>1/2</sub> term= half-life associated with the terminal phase of the concentration vs. time profile.<sup>f</sup> Area under the concentration versus time curve (extrapolated to infinity).<sup>g</sup> CL= serum clearance.<sup>h</sup> MRT= Mean residence time.

The initial volume of distribution approximated the plasma volume for both the Fab' and F(ab')<sub>2</sub>. Pegylation decreased serum CL of anti-IL8 fragments and extended both the terminal half-life and MRT as shown in Table 10 below.

**Table 10. Fold decrease/increase in clearance, terminal half-life & MRT of pegylated anti-IL8 fragments.**

anti-IL8 fragment	Fab'					F(ab') <sub>2</sub>		
Group No.	1	2	8	3	6	5	4	7
PEG structure	—	linear	linear	linear	bran.	—	bran.	bran.
No. of PEGs	—	1	1	1	1	—	1	2
PEG MW	—	20K	30K	40K	40K	—	40K	40K
CL : mean (mL/hr/kg)	110	2.5	2.2	1.3	0.63	14	0.92	0.83
fold decrease	1	46	51	90	180	1	15	17
t <sub>1/2</sub> term : mean (hr)	3.0	44	43	50	110	8.5	45	48
fold increase	1	14	14	17	35	1	5.3	5.7
MRT : mean (hr)	0.61	32	45	63	140	4.2	55	64
fold increase	1	53	73	100	240	1	13	15

5

For the pegylated anti-IL8 Fab' fragments, CL decreased by 46 to 180-fold. Terminal half-life and MRT increased 14 to 35-fold and 53 to 240-fold, respectively. For pegylated anti-IL8 F(ab')<sub>2</sub> molecules, CL decreased 15 to 17-fold with pegylation, and terminal half-life and MRT increased by greater than 5-fold and 13-fold, respectively. The changes in these parameters increased for both pegylated Fab' and F(ab')<sub>2</sub> molecules with increasing PEG molecular weight and approached the values of the full-length anti-IL8 (terminal half-life of 74 hours, MRT of 99 hours and CL of 0.47 mL/hr/kg). In comparing the branched(1)40K Fab' (Group 6) and branched(1)40K F(ab')<sub>2</sub> (Group 4), unexpected pharmacokinetics were observed. The pegylated Fab' molecule appeared to remain in the serum longer than the pegylated F(ab')<sub>2</sub> (see Figure 66). The mean CL of branched(1)40K Fab' was 0.63 mL/hr/kg, but a higher CL was observed for branched(1)40kD F(ab')<sub>2</sub> (CL 0.92 mL/hr/kg). The terminal half-life, likewise, was longer for the Fab' than the F(ab')<sub>2</sub> pegylated molecule (110 vs 45 hours).

The pharmacokinetic data demonstrated that pegylation decreased CL and increased terminal t<sub>1/2</sub> and MRT of anti-IL8 fragments (Fab' and F(ab')<sub>2</sub>) to approach that of the full-length anti-IL8. Clearance was decreased with pegylation 46 to 180-fold for the Fab' and approximately 16-fold for the F(ab')<sub>2</sub>. The terminal half-life of the Fab' anti-IL8 fragment was increased by 14 to 35-fold and approximately 5-fold for the F(ab')<sub>2</sub> anti-IL8. MRT, likewise, were extended by 53 to 240-fold for the Fab' and approximately 14-fold for the F(ab')<sub>2</sub>. The

branched(1) 40kD Fab' had a longer terminal half-life and lower clearance compared to the branched(1) 40kD F(ab')<sub>2</sub>.

Y. IN VIVO EFFICACY TESTING OF ANTI-IL-8 ANTIBODY REAGENTS IN RABBIT  
 5 MODEL OF ISCHEMIA/REPERFUSION AND ACID ASPIRATION-INDUCED ACUTE  
RESPIRATORY DISTRESS SYNDROME (ARDS)

Full length murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5, 40 kD branched PEG-6G4V11N35A Fab', and control antibody (anti-HIV gp120 monoclonal antibody 9E3.1F10) were tested in a rabbit ARDS model. The animals were weighed and anaesthetized by  
 10 intramuscular injection of ketamine (50 mg/kg body weight), xylazine (5 mg/kg body weight), and acepromazine (0.75 mg/kg body weight). A second dose (20% of the first dosage) was given IM 15 minutes before removal of vascular clip, and third dose (60% of the first dosage) was given at tracheotomy. Intra-arterial catheter (22G, 1 in. Angiocath) and intra-venous catheter (24G, 1 in. angiocath) were be placed in the ear central artery and posterior marginal ear vein for  
 5 blood samplings (arterial blood gases and CBC) and anti-IL-8 and fluid administration, respectively. The anaesthetized animals were transferred in a supine position to an operating tray; the abdominal area was shaved and prepared for surgery. Via a midline laparotomy, the superior mesenteric artery (SMA) was isolated and a microvascular arterial clip applied at the aortic origin. Before the temporary closure of the abdomen using 9 mm wound clip (Autoclip, Baxter), 15 ml of normal saline was given intraperitoneally as fluid supplement. After 110  
 20 minutes of intestinal ischemia, the abdominal incision was reopened and the arterial clip was released to allow reperfusion. Before closure, 5 ml of normal saline was given intraperitoneally for fluid replacement. The laparotomy incision was closed in two layers and the animals allowed to awaken.

25 After surgery, the animals were placed on a heating pad (38°C) and continuously monitored for up to 6 hours post reperfusion and lactated Ringer's 8-12 ml/kg/hr IV was given as fluid supplement.

At 22-24 hr post-reperfusion, a tracheotomy was performed under anesthesia. Normal physiologic saline was diluted 1:3 with water and adjusted to pH 1.5 (adjusted by using 1N HCL); 3 ml/kg body weight was then instilled intra-tracheally. Rectal temperature was maintained at 37 +/- 1 degree C using a homeothermic heat therapy pad (K-Mod II, Baxter).

5 Fluid supplements (LRS) at a rate of 5 ml/kg/hour IV were given. Blood gases were monitored every hour. The rabbits were returned to the cage after 6 hr of continuous monitoring.

Just prior to aspiration, animals were treated with saline, the control monoclonal antibody (anti-HIV gp-120 IgG 9E3.1F10), the full length murine anti-rabbit IL8 (6g4.2.5 murine IgG2a anti-rabbit IL8) or the pegylated 6G4V11N35A Fab' (6G4V1N35A Fab' modified with 40kD  
10 branched PEG-maleimide as described in Section T above, denoted as "40 kD branched PEG-6G4V11N35A Fab' "). Data from saline or control antibody treated animals was combined and presented as "Control". Arterial blood gases and A-a PO2 gradient measurements were taken daily, and IV fluid supplementation was performed daily. A-a PO2 gradient was measured at 96 hr of reperfusion. The A-a PO2 gradient was calculated as:

$$15 \quad A-a \text{ PO}_2 = [\text{FIO}_2(\text{PB} - \text{PH}_2\text{O}) - (\text{PaCO}_2/\text{RQ})] - \text{PaO}_2.$$

PaO2/FiO2 ratios were measured at 24hr and 48hr in room air and 100% oxygen.

After the final A-a PO2 gradient measurement, the animals were anesthetized with Nembutal 100mg/kg i.v. and the animals were euthanized by transecting the abdominal aorta in order to reduce red blood cell contamination of bronchoalveolar lavage fluid (BAL). The lungs were removed en bloc. The entire lung was weighed and then lavaged with an intratracheal tube (Hi-Lo tracheal tube, 3mm) using 30 ml of HBSS and lidocain. Total and differential leukocyte counts in the BAL were determined. Lesions/changes were verified by histological examination of each lobe of the right lung of each animal.

25 The gross lung weight, total leukocyte and polymorphonuclear cell counts in BAL, and PaO2/FiO2 data obtained are depicted in Figs. 67, 68 and 69, respectively. Treatment with 40 kD branched PEG-6G4V11N35A Fab' exhibited no effect on the biological parameters measured in the model as compared to the "Control" group. However, the data do not contradict the

pharmacokinetic analysis or the in vitro activity analysis for the 40 kD branched PEG-6G4V11N35A Fab' presented in Sections (V) and (X) above. In addition, these data do not contradict the ability of the 40 kD branched PEG-6G4V11N35A Fab' to reach and act on disease effector targets in circulation or other tissues.

5

Z. ADDITIONAL IN VIVO EFFICACY TESTING OF ANTI-IL-8 ANTIBODY REAGENTS IN RABBIT MODEL OF ISCHEMIA/REPERFUSION AND ACID ASPIRATION-INDUCED ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)

10 Full length murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 and 20 kD linear PEG-6G4V11N35E Fab' were tested in a rabbit model of ischemia/reperfusion- and acid aspiration-induced acute respiratory distress syndrome (ARDS).

**Antibodies**

15 A Fab'-SH antibody fragment of the affinity matured anti-IL-8 antibody 6G4V11N35E was expressed using the Fab' expression plasmid for 6G4V11N35E (described in Section (T) above) in *E. coli* grown to high density in the fermentor as described by Carter et al., Bio/Technology, 10: 163-167 (1992). Anti-IL-8 6G4V11N35E Fab' variant was purified from fermentation paste and modified with 20 kD linear methoxy-PEG-maleimide as described in Example T above. Pegylated material was formulated in phosphate buffered saline (PBS) at physiological pH. Full length 6G4.2.5 antibody was obtained from hybridoma cell line 6G4.2.5 as described in Section (B) above and formulated in phosphate buffered saline (PBS) at physiological pH.

20

**Sterile Surgical Procedures and Post-Operative Care**

25

Male New Zealand White rabbits weighing 2.2 to 2.5 kg (obtained from Western Oregon Rabbit Company) were anaesthetized by intramuscular injection of ketamine (50 mg/kg body weight), xylazine (5 mg/kg body weight), and acepromazine (0.75 mg/kg body weight). Intra-arterial catheter (22G, 1 in. Angiocath) and intra-venous catheter (24G, 1 in. angiocath) were be

placed in the ear central artery and posterior marginal ear vein for blood samplings (arterial blood gases and CBC) and anti-IL-8 (or fluid) administration, respectively. The anaesthetized animals were transferred in a supine position to an operating tray; the abdominal area was shaved and prepared for surgery. Via a midline laparotomy, the superior mesenteric artery (SMA) was isolated and a microvascular arterial clip applied at the aortic origin. Before the temporary closure of the abdomen using 9 mm wound clip (Autoclip, Baxter), 15 ml of normal saline (38°C) was given intraperitoneally as fluid supplement. After 110 minutes of intestinal ischemia, the abdominal incision was reopened and the arterial clip was released to allow reperfusion. Before closure, 5 ml of normal saline (38°C) was given intraperitoneally for fluid replacement.

The laparotomy incision was closed in two layers and the animals allowed to awaken.

After surgery, the animals were placed on a heating pad (38°C) and continuously monitored for up to 6 hours post reperfusion and lactated Ringer's 8-12 ml/kg/hr IV was given as fluid supplement.

At 22-24 hr post-reperfusion, a tracheotomy was performed under anesthesia using ketamine, xylazine and acepromazine as described above. Normal physiologic saline was diluted 1:3 with water and adjusted to pH 1.5 (adjusted by using 1N HCL), and 3 ml/kg body weight was then instilled intra-tracheally through an uncuffed tracheal tube (2.0mm I.D., Mallinckrodt Medical, Inc.). After instillation, the trachea was closed with 3-0 silk suture and the rabbits were allowed to recover. Rectal temperature was maintained at 37°C +/- 1°C using a homeothermic heat therapy pad (K-Mod II, Baxter). Fluid supplements (LRS) at a rate of 5 ml/kg/hour IV were given. The rabbits were observed and blood gases in room air and in 100% oxygen were measured daily.

### **Dose Administration**

Treated animals received an intravenous injection of 7 mg/kg 20 kD linear PEG-6G4V11N35E Fab' (n=5 animals) at 10 minutes before and 6 hours after acid instillation.



## Oxygenation Measurement

Alveolar-arterial oxygen pressure gradient (A-a PO<sub>2</sub> gradient) was calculated as follows:

$$A-a\ PO_2 = [FiO_2(PB - PH_2O) - (PaCO_2/RQ)] - PaO_2$$

where FiO<sub>2</sub> is fraction of inspired oxygen, PB is barometric pressure, PH<sub>2</sub>O is partial pressure of water vapor, PaCO<sub>2</sub> is arterial carbon dioxide pressure, RQ is respiratory quotient, and PaO<sub>2</sub> is arterial oxygen pressure.

A-a PO<sub>2</sub> gradient and PaO<sub>2</sub>/FiO<sub>2</sub> ratios for each rabbit were measured at baseline (pre-op), before acid instillation, every hour up to 6 hours after acid instillation, and every 24 hours thereafter.

## Bronchoalveolar Lavage (BAL)

After blood gases measurement at 72 hours post reperfusion, the rabbits were anesthetized with Nembutal 50 mg/kg i.v. and were euthanized by exsanguination. The abdominal aorta was transected to reduce red blood cell contamination of bronchial alveolar lavage fluid (BALF). The lung and heart were removed en bloc. The right lung was lavaged with an intratracheal tube (Hi-Lo tracheal tube, 3.0 mm) using 20 ml of HBSS and lidocain. Total and differential leukocyte counts of BALF were determined.

## Gross Lung Weight

The whole lung from each rabbit was weighed immediately after harvest and was expressed as g/kg of body weight.

## Peripheral Blood Count

Blood samples (0.05 ml for CBC, 0.2 ml for blood gases) were collected from the ear central artery catheter at baseline (pre-op), 2 hours, 4 hours, 6 hours, and 22 hours post reperfusion (prior to acid or saline instillation) and at 1 hour, 2 hours, 3 hours, 4 hours, 6 hours and every 24 hours after acid instillation. Hematology parameters were determined by Automated Hematology Analyzer according to the standard hematological procedures.

## Pharmacokinetics

Blood samples (0.5 ml) were collected from the ear central artery catheter at baseline (pre-op), 4 hours, and 22 hours post reperfusion and at 1 hour, 4 hours, and every 24 hours after acid instillation.

5

## Results and Discussion

In the rabbit model of ARDS, lung injury is manifested by hypoxemia (low PaO<sub>2</sub> - the pressure of O<sub>2</sub> in the arterial blood, as measured by a blood gas machine), lung edema (evidenced by an elevated lung weight to body weight ratio) and pro-inflammatory infiltrates into the alveolar space (evidenced by high white blood cell (WBC) and neutrophil (PMN) numbers). Although 40 kD branched PEG-6G4V11N35A Fab' did not protect rabbits from lung injury at any of the doses tried (5 mg/kg and 20 mg/kg) (see Section (Y) above), the 20 kD linear PEG-6G4V11N35E Fab' had efficacy equal to, and, for some end-points, superior to that of the full length IgG murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 and prevented lung injury in the rabbits as shown in Figs. 70A-70E. (The data points for 40 kD branched PEG-6G4V11N35A Fab' treated animals, full length 6G4.2.5 treated animals, and saline treated animals appearing in Figs. 70A-70E are taken from the data displayed in Figs. 67-69 and generated in Example Y above.) In addition, these data indicate that large effective size anti-IL-8 Fab'-PEG conjugates can exhibit useful levels of efficacy in acute lung injury and ARDS.

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### AA. IN VIVO EFFICACY TESTING OF ANTI-IL-8 ANTIBODY REAGENTS IN RABBIT EAR MODEL OF TISSUE ISCHEMIA AND REPERFUSION

Full length murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5, 20 kD linear PEG-6G4V11N35E Fab', 30 kD linear PEG-6G4V11N35E Fab', and 40 kD branched PEG-6G4V11N35E Fab' were tested in a rabbit ear model of tissue ischemia and reperfusion injury.

25

## Antibodies

A Fab'-SH antibody fragment of the affinity matured anti-IL-8 antibody 6G4V11N35E

was expressed using the Fab' expression plasmid for 6G4V11N35E (described in Example T above) in *E. coli* grown to high density in the fermentor as described by Carter et al., Bio/Technology, 10: 163-167 (1992). Anti-IL-8 6G4V11N35E Fab' variant was purified from fermentation paste and modified with 20 kD linear methoxy-PEG-maleimide, 30 kD linear methoxy-PEG-maleimide, or 40 kD branched methoxy-PEG-maleimide as described in Example T above. Pegylated material was formulated in phosphate buffered saline (PBS) at physiological pH.

### Animals

1.0 to 1.5 kg New Zealand White rabbits were obtained from Western Oregon Rabbit Company.

### Surgical procedure and animal evaluation

The procedure was essentially described by Vedder et al., Proc. Natl. Acad. Sci. (USA), 87: 2643-2646 (1990). Briefly, general anesthesia was achieved by intramuscular injections of Ketamine (50 mg/kg) plus Xylazine (5 mg/kg) and Acepromazine (2 mg/kg). The right external ear was prepared for surgery and under sterile procedure the ear was transected at its base, leaving intact only the central artery and vein. All nerves were transected to ensure that the ear was completely anesthetic. A straight microaneurysm clip (1.5x10mm) was placed across the artery to produce complete ischemia. The ear was reattached with the clip exiting through the wound. The rabbits were then housed at 26°C and 6 hours later the clip was removed to effect reperfusion. Untreated rabbits (n=11 animals) received an intravenous injection of vehicle (10 mM sodium acetate, 8% trehalose and 0.01% polysorbate-20 at pH 5.5) immediately prior to reperfusion. Treated animals received 5 mg/kg full length IgG murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 (n=4 animals), 20 kD linear PEG-6G4V11N35E Fab' (n=3 animals), 30 kD linear PEG-6G4V11N35E Fab' (n=3 animals), or 40 kD branched PEG-6G4V11N35E Fab' (n=3 animals) immediately prior to reperfusion.

The ear volume and necrosis were measured daily by procedures described in Vedder et al., supra. Briefly, the ear was submerged in a beaker of water containing 1.2% Povidone iodine (Baxter) up to the intertragic incisure and the ear volume determined by the volume of fluid displaced. The ears were monitored in this manner for 7 days. The data are represented (in Fig. 71) as percent change in ear volume calculated as follows:

$$\% \text{ change in ear volume} = \frac{(\text{Ear vol. at day } x - \text{Ear vol. at day } 0) \times 100\%}{\text{Ear vol. at day } 0}$$

Animals were sacrificed at day 1 and day 7 for histological evaluation of the ear and the same section of ear was taken from all animals.

To determine that the therapeutic agents did not adversely affect any hematological parameter, aliquots of blood were withdrawn for complete blood counts and differentials immediately before reperfusion and at 24 hour intervals. In a separate experiment, blood samples were taken at 1, 5, 15, and 30 minutes and at 1 hour and 4 hours.

## Results and Discussion

In the rabbit model of ear ischemia reperfusion injury, antibody was administered intravenously at a single dose (5 mg/kg) at the time of reperfusion. In this model, ischemia reperfusion injury is characterized by tissue damage, edema and sometimes necrosis; all attributable in part to neutrophil-mediated damage. Monitoring of ear volume over time is a surrogate end-point for evaluating edema in the ear tissue. The resulting data (depicted in Fig. 71) showed that treatment with 20 kD linear PEG-, 30 kD linear PEG- and 40 kD branched PEG-conjugated Fab's effectively reduced ear swelling and edema at all time points of observation (days 1, 3 and 5). In fact, the efficacy of all three PEGylated Fab's was statistically indistinguishable from that of the full length IgG murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 at all time points observed. These data support the efficacy of large effective size anti-IL-8 Fab'-PEG conjugates in ischemic reperfusion injury and specifically support the ability of

40 kD branched PEG-conjugated Fab' molecules to reach and act on disease effector targets in circulation and other tissues.

AB. PHARMACOKINETIC STUDIES OF TWO CONSTRUCTS OF PEGYLATED ANTI-VEGF FAB' FRAGMENTS FOLLOWING INTRAVENOUS AND INTRAPERITONEAL ADMINISTRATION IN NORMAL MICE

The objective of this study was to characterize and compare the pharmacokinetics of two pegylated species of the Y0317 affinity matured variant of humanized anti-VEGF F(ab)-12 (Y0317 is described in WO 98/45331 published October 15, 1998) (International Application No. PCT/US98/06604 filed April 3, 1998) when administered intravenously and intraperitoneally in normal mice.

**Methods**

The Y0317 anti-VEGF Fab' was obtained as described in Example 3 of WO 98/45331. The Y0317 anti-VEGF Fab' was pegylated with 20 kD linear PEG or 40 kD branched PEG using the thiol protection/deprotection maleimide coupling method described in Example T above to form 20 kD linear PEG-Y0317 Fab' and 40 kD branched PEG-Y0317 Fab', respectively. Pegylated Fab's were formulated in phosphate buffered saline (PBS) at physiological pH.

Male CD-1 mice (Charles River Laboratories, Hollister, CA), weighing between 18.9 and 28.8 grams, were injected with a single intravenous (IV) or intraperitoneal (IP) dose of either 20 kD linear or 40kD branched PEG-Y0317 Fab' at approximately 3 mg protein/kg. Blood was collected via cardiac puncture upon terminal sacrifice at the following time points (n=2 per time point): pre-dose, 5 and 30 minutes; 1, 2, 4, 8, 24, 32 hours; Days 2, 3, 5, 7 10 and 14. Serum was harvested and analyzed for PEGylated Y0317 Fab' concentrations by ELISA. In addition, approximately 0.2 mL of serum was collected pre-dose from the Day 7 and 14 mice (orbital bleed) as controls for potential antibody analysis.

For each PEGylated Y0317 Fab', the pooled individual serum PEGylated Y0317 Fab' concentration data following IV and IP administration were analyzed using a two-compartment

and one-compartment pharmacokinetic model, respectively. Concentration values that were below the lower limit of the assay were not used in the analysis.

## Results

Table 11 below summarizes the compartmental pharmacokinetic parameters of the 20 kD linear and 40 kD branched PEG-Y0317 Fab'. Figures 1 and 2 display the serum concentration versus time profiles following IV and IP administration, respectively, for the 20 kD linear and 40 kD branched PEG-Y0317 Fab'.

Table 11: Summary of PK parameters

	20 K		40K	
	IV	IP	IV	IP
Dose (mg/kg)	3	3	3.4	3.4
C <sub>max</sub> (μg/mL)	58.1	11.5	74.6	49.8
T <sub>max</sub>	5 min	8 hr	30 min	4 hr
CL (mL/day/kg)	179	236	49.4	37.0
t <sub>1/2</sub> α (hr)	1.34	--	1.78	--
t <sub>1/2</sub> β (hr)	16.6	16.0 <sup>a</sup>	29.8	28.3 <sup>a</sup>
AUC (day•μg/mL)	16.8	12.7	68.9	91.8
V <sub>ss</sub> (mL/kg)	139	228	82.8	63.3
BA (%)	--	75.6	--	133

<sup>a</sup>K10 half-life was reported for IP.

After intravenous administration, the clearance of the 20 kD linear PEG-Y0317 Fab' was 179 mL/day/kg and decreased to 49.4 mL/day/kg with the 40 kD branched PEG-Y0317 Fab'.

V<sub>ss</sub>, also decreasing with a larger PEG size, was 139 and 82.8 for the 20 kD and 40 kD

PEGylated species, respectively. In accord with the decrease in clearance and volume of distribution, an increased terminal half-life was observed for the larger PEG size (terminal t<sub>1/2</sub> of

approximately 17 and 30 hours for the 20 kD and 40 kD PEGylated species, respectively). After IP administration, the bioavailability was approximately 76 and 133% for the 20 kD and 40 kD PEGylated species, respectively. Results from previous pharmacokinetic studies with intravenous administration of an anti-CD18 Fab in a normal mouse model (Zapata et al., "Site Specific Coupling of Monomethoxypoly(ethylene) glycol to a single-sulfhydryl humanized Fab", poster presented at the American Society for Biochemistry and Molecular Biology FASEB Meeting held in San Francisco, CA on May 21-25, 1995; Abstract No. 1288 published in Zapata et al., FASEB J., 9(6): A1479 (1995)) indicated that the clearance of a non-PEGylated Fab' was approximately 4500 mL/day/kg and the terminal half-life was approximately 30 minutes. Taken together, these data indicate that 20 kD (linear) and 40 kD (branched) PEGylation of the Y0317 Fab' resulted in an approximately 25-fold to 90-fold decrease in clearance and a 29-fold and 52-fold increase in terminal  $t_{1/2}$ , respectively.

#### AC. IN VIVO EFFICACY TESTING OF ANTI-VEGF ANTIBODY REAGENTS IN MOUSE MODEL OF TUMOR GROWTH

40 kD branched PEG-Y0317 anti-human VEGF Fab' was tested in a mouse tumor growth model.

#### **Methods**

40 kD branched PEG-Y0317 anti-human VEGF Fab' was obtained as described in Example AB above. Y0317 anti-human VEGF MAbs (full length IgG1) was obtained by fusing the Y0317 variable light (VL) and variable heavy (VH) domain sequences to constant light (CL) and constant heavy (CH) domain sequences, respectively, in separate pRK expression vectors as described in Eaton et al., Biochemistry, 25: 8343-8347 (1986), co-transfecting the expression constructs into 293 cells or CHO cells, and harvesting antibody from transfected cell culture supernatant essentially as described in Example 1 of WO 98/45331 (published October 15, 1998) (International Application No. PCT/US98/06604 filed April 3, 1998). Control 40 kD branched PEG-6G4V11N35E anti-rabbit IL-8 Fab' was obtained as described in Example AA above.

Human A673 rhabdomyosarcoma cells (ATCC; CRL 1598) were cultured as previously described in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics (Kim *et al. Nature* 362:841-844 (1993) and Borgström *et al. Cancer Res.* 56:4032-4039 (1996)). Female Beige nude mice, 6-10 weeks old (Harlan Sprague Dawley), were injected subcutaneously in the dorsal area with  $2.5 \times 10^6$  A673 tumor cells in a volume of 100  $\mu$ l of matrigel. Animals were then treated with 40 kD branched PEG-Y0317 Fab', Y0317 MAb, control 40 kD branched PEG-6G4V11N35E anti-rabbit IL-8 Fab', or phosphate buffered saline (PBS) at physiological pH.

In the low dose 40 kD branched PEG-Y0317 Fab' treatment group, 40 kD branched PEG-Y0317 Fab' was administered at a loading dose of 2 mg/kg on day 1, followed by maintenance doses of 0.9 mg/kg daily for the remainder of the study.

In the high dose 40 kD branched PEG-Y0317 Fab' treatment group, 40 kD branched PEG-Y0317 Fab' was administered at a loading dose of 6 mg/kg on day 1, followed by maintenance doses of 2.7 mg/kg daily for the remainder of the study.

In the Y0317 MAb treatment group, Y0317 MAb was administered at a loading dose of 8 mg/kg on day 1, followed by maintenance doses of 0.8 mg/kg every third day for the remainder of the study.

In the control Fab' treatment group, 40 kD branched PEG-6G4V11N35E anti-rabbit IL-8 Fab' was administered at a loading dose of 6 mg/kg on day 1, followed by maintenance doses of 2.7 mg/kg daily for the remainder of the study.

In the PBS control group, 0.1 ml/day of PBS was administered for the duration of the study. All doses were administered intraperitoneally in a volume of 100  $\mu$ l, starting 24 hr after tumor cell inoculation.

Each group initially consisted of 10 mice. Tumor size (length x width x height) was determined at weekly intervals. 17 days after tumor cell inoculation, animals were euthanized and the tumors were removed and weighed. Statistical analysis was performed by ANOVA.



## Results

As shown in Fig. 74, at both doses tested (2 and 6 mg/kg), the 40 kD branched PEG-Y0317 Fab' markedly suppressed tumor growth as assessed by tumor weight measurements three weeks after tumor cell inoculation. The decreases were 91% and 90%, respectively, in animals treated with the low and high doses of 40 kD branched PEG-Y0317 Fab' versus 95% in animals treated with Y0317 MAb.

The following biological materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

<u>Material</u>	<u>ATCC Accession No.</u>	<u>Deposit Date</u>
hybridoma cell line 5.12.14	HB 11553	February 15, 1993
hybridoma cell line 6G4.2.5	HB 11722	September 28, 1994
pantiIL-8.2, E. coli strain 294 mm	97056	February 10, 1995
p6G425chim2, E. coli strain 294 mm	97055	February 10, 1995
p6G4V11N35A.F(ab') <sub>2</sub>	97890	February 20, 1997
E. coli strain 49D6(p6G4V11N35A.F(ab') <sub>2</sub> )	98332	February 20, 1997
p6G425V11N35A.choSD	209552	December 16, 1997
clone#1933 aIL8.92 NB 28605/12	CRL-12444	December 11, 1997
clone#1934 aIL8.42 NB 28605/14	CRL-12445	December 11, 1997

These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable deposit for 30 years from the date of deposit. These cell lines will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the cell lines to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the cell lines to one determined by

the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

5 The assignee of the present application has agreed that if the deposited cell lines should be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a specimen of the same cell line. Availability of the deposited cell lines is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

WE CLAIM:

1. A conjugate consisting essentially of one or more antibody fragments covalently attached to one or more nonproteinaceous polymer molecules, wherein the apparent size of the conjugate is at least about 500 kD, and wherein at least one antibody fragment comprises an antigen binding site that binds to a polypeptide selected from the group consisting of: human vascular endothelial growth factor (VEGF), human p185 receptor-like tyrosine kinase (HER2), human CD20, human CD18, human CD11a, human IgE, human Apo-2 receptor, human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), human tissue factor (TF), human  $\alpha 4\beta 7$  integrin, human GPIIb-IIIa integrin, human epidermal growth factor receptor (EGFR), human CD3, and human interleukin-2 receptor  $\alpha$ -chain (TAC).

2. The conjugate of claim 1, wherein the apparent size of the conjugate is at least about 800 kD.

3. The conjugate of claim 1, wherein the apparent size of the conjugate is at least about 1,400 kD.

4. The conjugate of claim 1, wherein the apparent size of the conjugate is at least about 1,800 kD.

5. The conjugate of claim 1, wherein the apparent size of the conjugate is at least about 8 fold greater than the apparent size of at least one antibody fragment.

6. The conjugate of claim 5, wherein the apparent size of the conjugate is at least about 15 fold greater than the apparent size of at least one antibody fragment.

7. The conjugate of claim 6, wherein the apparent size of the conjugate is at least about 25 fold greater than the apparent size of at least one antibody fragment.

8. The conjugate of claim 1, wherein the conjugate contains no more than one antibody fragment, and wherein the antibody fragment is selected from the group consisting of Fab, Fab', Fab'-SH, Fv, scFv and F(ab')<sub>2</sub>.

9. The conjugate of claim 8 wherein the antibody fragment is F(ab')<sub>2</sub>.

10. The conjugate of claim 1 wherein at least one antibody fragment is covalently attached to no more than about 10 nonproteinaceous polymer molecules.

11. The conjugate of claim 10 wherein the antibody fragment is covalently attached to no more than about 5 nonproteinaceous polymer molecules.

12. The conjugate of claim 11 wherein the antibody fragment is covalently attached to no more than about 2 nonproteinaceous polymer molecules.

13. The conjugate of claim 12 wherein the antibody fragment is attached to no more than 1 nonproteinaceous polymer molecule.

14. The conjugate of claim 12, wherein the antibody fragment comprises a heavy chain and a light chain derived from a parental antibody, wherein in the parental antibody the heavy and light chains are covalently linked by a disulfide bond between a cysteine residue in the light chain and a cysteine residue in the heavy chain, wherein in the antibody fragment the cysteine residue in the light or heavy chain is substituted with another amino acid and the cysteine residue in the opposite chain is covalently linked to a nonproteinaceous polymer molecule.

15. The conjugate of claim 8 wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH.

5 16. The conjugate of claim 15 wherein the antibody fragment is covalently attached to no more than 1 nonproteinaceous polymer molecule.

17. The conjugate of claim 16 wherein the nonproteinaceous polymer molecule in the conjugate is covalently attached to the hinge region of the antibody fragment.

10 18. The conjugate of claim 1 wherein at least one nonproteinaceous polymer is a polyethylene glycol (PEG).

15 19. The conjugate of claim 18 wherein the PEG has an average molecular weight of at least about 20 kD.

20 20. The conjugate of claim 19 wherein the PEG has an average molecular weight of at least about 40 kD.

21. The conjugate of claim 19 wherein the PEG is a single chain molecule.

22. The conjugate of claim 20 wherein the PEG is a branched chain molecule.

25 23. The conjugate of claim 19, wherein the conjugate contains no more than one antibody fragment, and wherein the antibody fragment is a F(ab')<sub>2</sub> and is covalently attached to no more than about 2 PEG molecules.

24. The conjugate of claim 19, wherein the conjugate contains no more than one antibody fragment, and wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH and is covalently attached to no more than one PEG molecule.

25. The conjugate of claim 24 wherein the PEG molecule is covalently attached to the hinge region of the antibody fragment.

26. The conjugate of claim 1, wherein the conjugate contains no more than one antibody fragment, wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH, wherein the antibody fragment is covalently attached to no more than one nonproteinaceous polymer molecule, and wherein the nonproteinaceous polymer molecule is a polyethylene glycol (PEG) having an average molecular weight of at least about 20 kD.

27. The conjugate of claim 26 wherein the PEG has an average molecular weight of at least about 30 kD.

28. The conjugate of claim 27 wherein the PEG has an average molecular weight of at least about 40 kD.

29. The conjugate of claim 26 wherein the PEG is a single chain molecule.

30. The conjugate of claim 28 wherein the PEG is a branched chain molecule.

31. The conjugate of claim 1 wherein the antibody fragment comprising the antigen binding site is humanized.

32. The conjugate of claim 1 wherein the conjugate contains no more than one antibody fragment.

33. The conjugate of claim 1, wherein the covalent structure of the conjugate is free of any matter other than the antibody fragment and nonproteinaceous polymer molecules that form the conjugate.

5

34. The conjugate of claim 1, wherein the covalent structure of the conjugate incorporates one or more nonproteinaceous labels, and wherein the covalent structure of the conjugate is free of any matter other than the antibody fragment, nonproteinaceous polymer and nonproteinaceous label molecules that form the conjugate.

10

35. The conjugate of claim 34 wherein at least one nonproteinaceous label is a radiolabel.

36. A composition comprising the conjugate of claim 1 and a carrier.

15

37. The composition of claim 36 that is sterile.

38. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human VEGF.

20

39. A method for inhibiting angiogenesis in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 38.

40. A method for treating a neovascular disorder in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 38.

25

41. The method of claim 40 wherein the neovascular disorder is tumor vascularization.

42. The method of claim 40 wherein the neovascular disorder is an intraocular neovascular disorder.

5 43. The method of claim 42 wherein the intraocular neovascular disorder is age-related macular degeneration (AMD).

44. A method for inhibiting the growth of tumor cells in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 38.

10 45. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to HER2.

15 46. A method for inhibiting the growth of a HER-2 expressing cancer cell in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 45.

47. The method of claim 46 wherein the cancer cell overexpresses HER2.

20 48. A method of treating a patient having a tumor that overexpresses HER2 comprising administering to the patient the conjugate of claim 45 in an amount effective to reduce the patient's tumor burden.

49. The method of claim 48 wherein the tumor is a breast cancer.



50. A method of binding the conjugate of claim 45 to a human cell expressing HER2, comprising contacting the cell with the conjugate under conditions wherein the conjugate binds to HER2 on the cell surface.

5 51. The method of claim 50 wherein the cell is a tumor cell.

52. The method of claim 51 wherein the tumor cell overexpresses HER2.

10 53. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human CD20.

15 54. A method of inhibiting the growth of a neoplastic cell expressing CD20 in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 54.

55. The method of claim 54 wherein the cell is a B lymphocyte.

20 56. A method of treating a CD20-expressing lymphoma in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 53.

57. The method of claim 56 wherein the lymphoma is a B lymphocytic lymphoma.

25 58. A method of binding the conjugate of claim 53 to a human cell expressing CD20, comprising contacting the cell with the conjugate under conditions wherein the conjugate binds to CD20 on the cell surface.

59. The method of claim 58 wherein the cell is a B lymphocyte.

60. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human CD18.

61. A method of treating an inflammatory disorder in a mammal comprising  
5 administering to the mammal an effective amount of the conjugate of claim 60.

62. The method of claim 61 wherein the inflammatory disorder is an ischemic reperfusion disorder.

10 63. The method of claim 62 wherein the ischemic reperfusion disorder is acute myocardial infarction.

64. The method of claim 62 wherein the ischemic reperfusion disorder is stroke.

15 65. A method of treating an immune disorder in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 60.

66. The method of claim 65 wherein the immune disorder is graft rejection in a transplant recipient.

20 67. A method of binding the conjugate of claim 60 to a human cell expressing CD18, comprising contacting the cell with the conjugate under conditions wherein the conjugate binds to CD18 on the cell surface.

25 68. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human CD11a.

69. A method of treating an inflammatory disorder in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 68.

70. The method of claim 69 wherein the inflammatory disorder is psoriasis.

71. A method of treating an immune disorder in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 68.

72. The method of claim 71 wherein the immune disorder is graft rejection in a transplant recipient.

73. The method of claim 71 wherein the immune disorder is multiple sclerosis.

74. A method of treating asthma in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 68.

75. A method of binding the conjugate of claim 68 to a human cell expressing CD11a comprising contacting the cell with the conjugate under conditions wherein the conjugate binds to CD11a on the cell surface.

76. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human IgE.

77. The conjugate of claim 76 comprising at least one antibody fragment having an antigen binding site that binds to membrane-bound IgE on human B-lymphocytes but does not bind to soluble IgE bound to Fc $\epsilon$ RI receptor on human basophils.

78. A method of treating an IgE-mediated disorder in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 77.

79. The method of claim 78 wherein the IgE-mediated disorder is an allergic disease.

80. The method of claim 79 wherein the allergic disease is allergic rhinitis.

81. The method of claim 79 wherein the allergic disease is allergic asthma.

82. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human Apo-2 receptor.

83. The conjugate of claim 82 wherein the anti-human Apo-2 receptor antibody fragment is an Apo-2 receptor agonist.

84. A method of treating cancer in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 83.

85. The method of claim 84 wherein the cancer is colon cancer.

86. A method for inducing apoptosis of a human cell expressing Apo-2 receptor comprising contacting the cell with the conjugate of claim 83 under conditions wherein the conjugate induces apoptotic death of the cell.

87. The method of claim 86 wherein the cell is a cancer cell.

88. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human TNF- $\alpha$ .

89. A method of treating an inflammatory disorder in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 88.

5 90. The method of claim 89 wherein the inflammatory disorder is Crohn's disease.

91. The method of claim 89 wherein the inflammatory disorder is inflammatory bowel disease.

10 92. The method of claim 89 wherein the inflammatory disorder is rheumatoid arthritis.

15 93. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human tissue factor.

20 94. A method for treating a thrombotic disease in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 93.

95. The method of claim 94 wherein the thrombotic disease is deep vein thrombosis.

96. The method of claim 94 wherein the thrombotic disease is arterial thrombosis.

25 97. A method for inhibiting blood coagulation in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 93.

98. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human GPIIb-IIIa integrin.

99. A method for treating a thrombotic disorder in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 98.

100. The method of claim 99 wherein the thrombotic disorder is vascular restenosis.

101. The method of claim 99 wherein the thrombotic disorder is unstable angina.

102. A method of binding the conjugate of claim 98 to a human cell expressing GPIIb-IIIa integrin comprising contacting the cell with the conjugate under conditions wherein the conjugate binds to the cell.

103. The method of claim 102 wherein the cell is a platelet.

104. A method for inhibiting platelet aggregation in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 98.

105. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human  $\alpha_4\beta_7$  integrin.

106. A method of treating an inflammatory disorder in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 105.

107. The method of claim 106 wherein the inflammatory disorder is inflammatory bowel disease (IBD).

108. A method of binding the conjugate of claim 105 to a human cell expressing  $\alpha_4\beta_7$  comprising contacting the cell with the conjugate under conditions wherein the conjugate binds to the cell.

109. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human EGFR.

5 110. A method of treating cancer in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 109.

111. A method of inhibiting the growth of a cancer cell in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 109.

10 112. A method of binding the conjugate of claim 109 to a human cell expressing EGFR comprising contacting the cell with the conjugate under conditions wherein the conjugate binds to the cell.

15 113. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human CD3.

20 114. A method of treating an immune disorder in a mammal administering to the mammal an effective amount of the conjugate of claim 113.

115. The method of claim 114 wherein the immune disorder is graft rejection in a transplant recipient.

25 116. A method of binding the conjugate of claim 113 to a human cell expressing CD3 comprising contacting the cell with the conjugate under conditions wherein the conjugate binds to the cell.

117. The method of claim 116 wherein the cell is a T lymphocyte.

118. The conjugate of any of claims 1-35 comprising at least one antibody fragment having an antigen binding site that binds to human interleukin-2 receptor  $\alpha$ -chain (TAC).

5 119. A method of treating an immune disorder in a mammal comprising administering to the mammal an effective amount of the conjugate of claim 118.

120. The method of claim 119 wherein the immune disorder is graft rejection in a transplant recipient.

10

121. The method of claim 120 wherein the graft rejection is kidney graft rejection.

15

122. A method of binding the conjugate of claim 118 to a human cell expressing TAC comprising contacting the cell with the conjugate under conditions wherein the conjugate binds to the cell.

123. The method of claim 122 wherein the cell is a T or B lymphocyte.

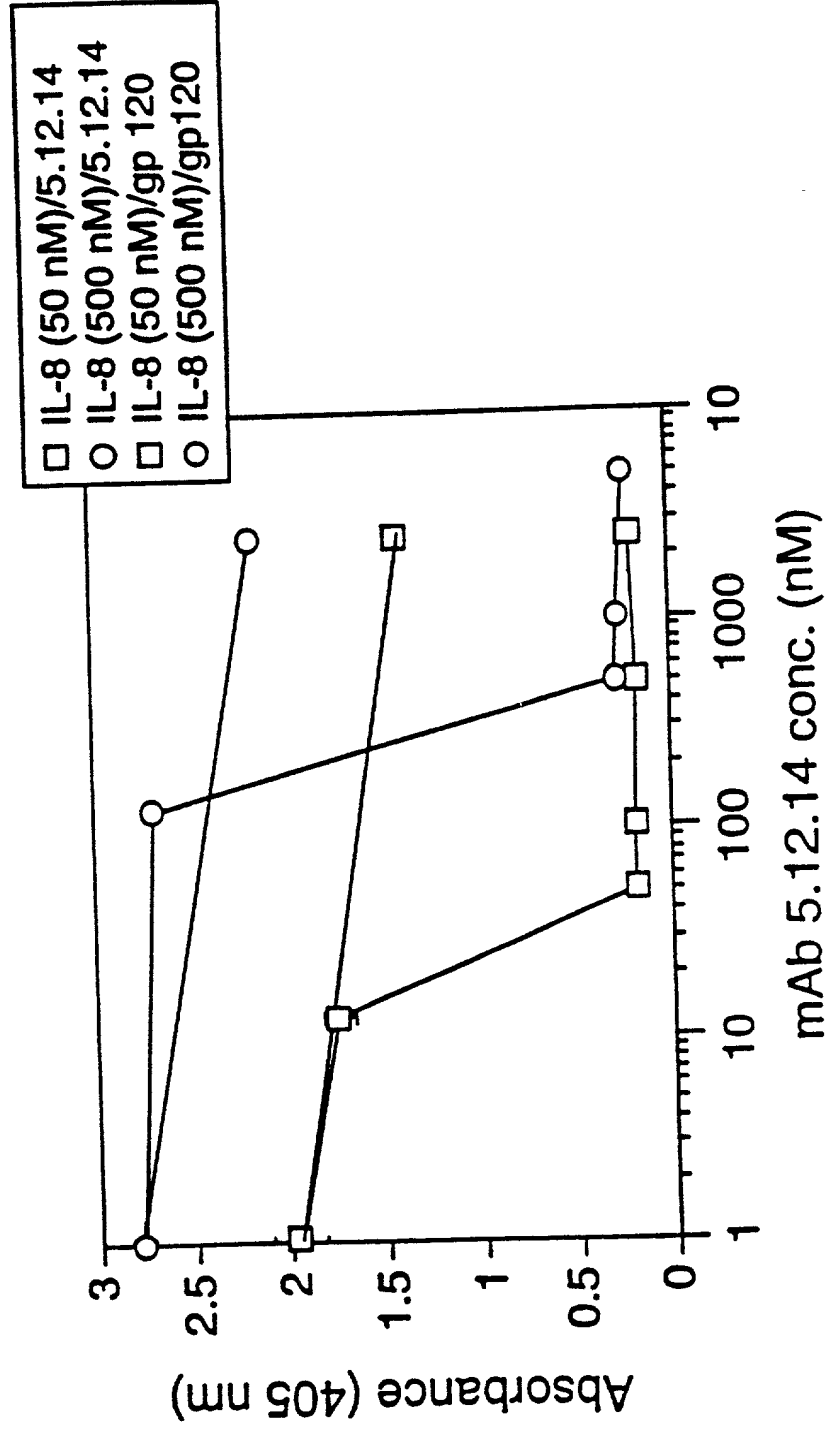


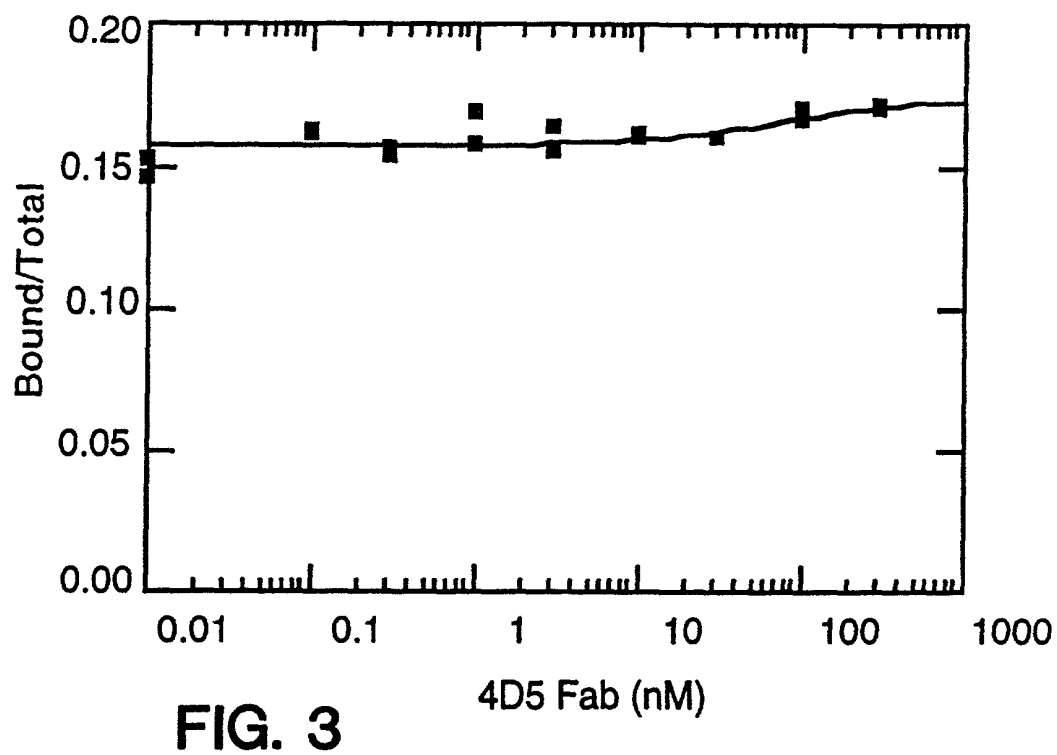
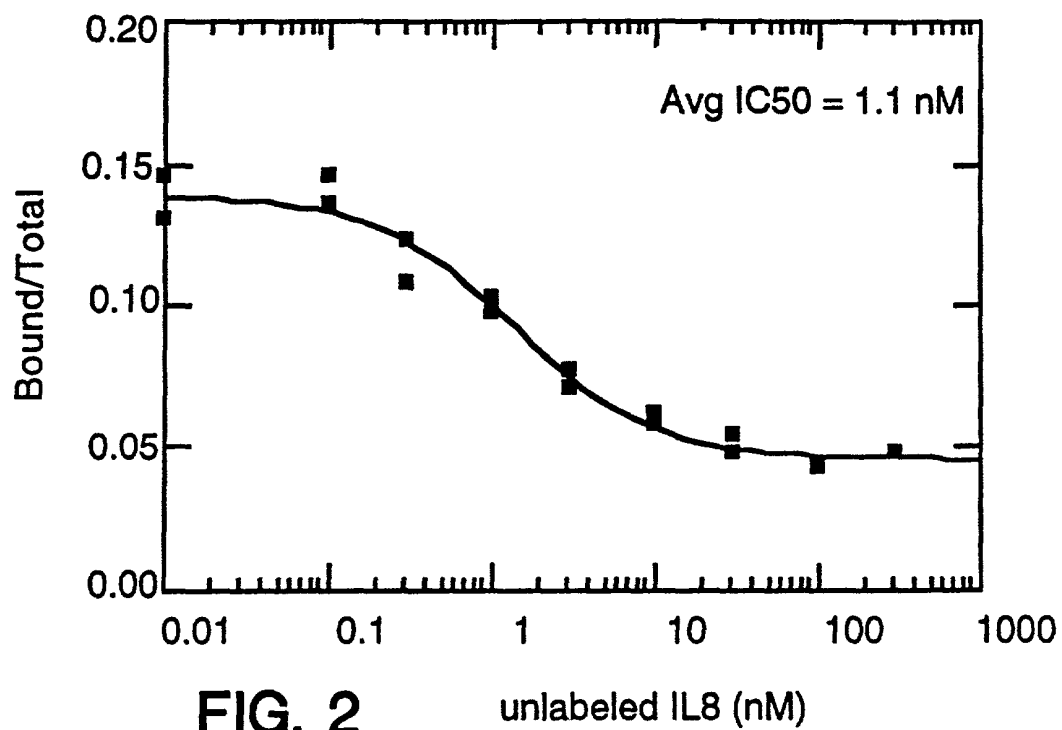
# ANTIBODY FRAGMENT-POLYMER CONJUGATES AND USES OF SAME

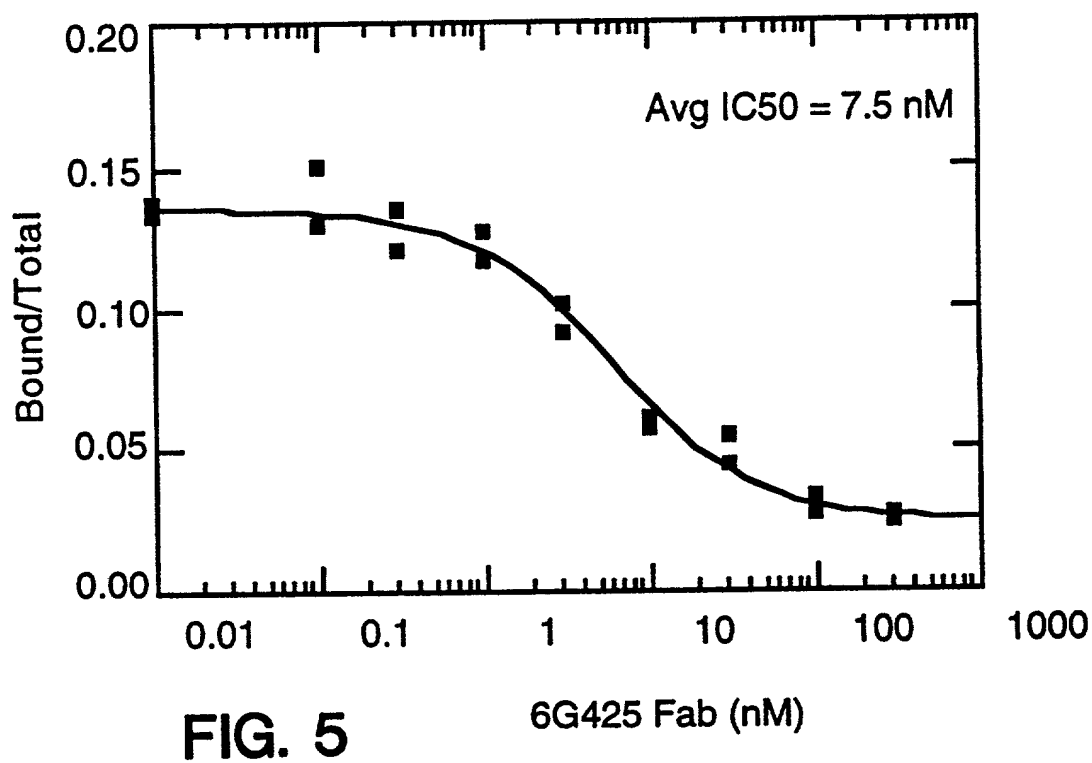
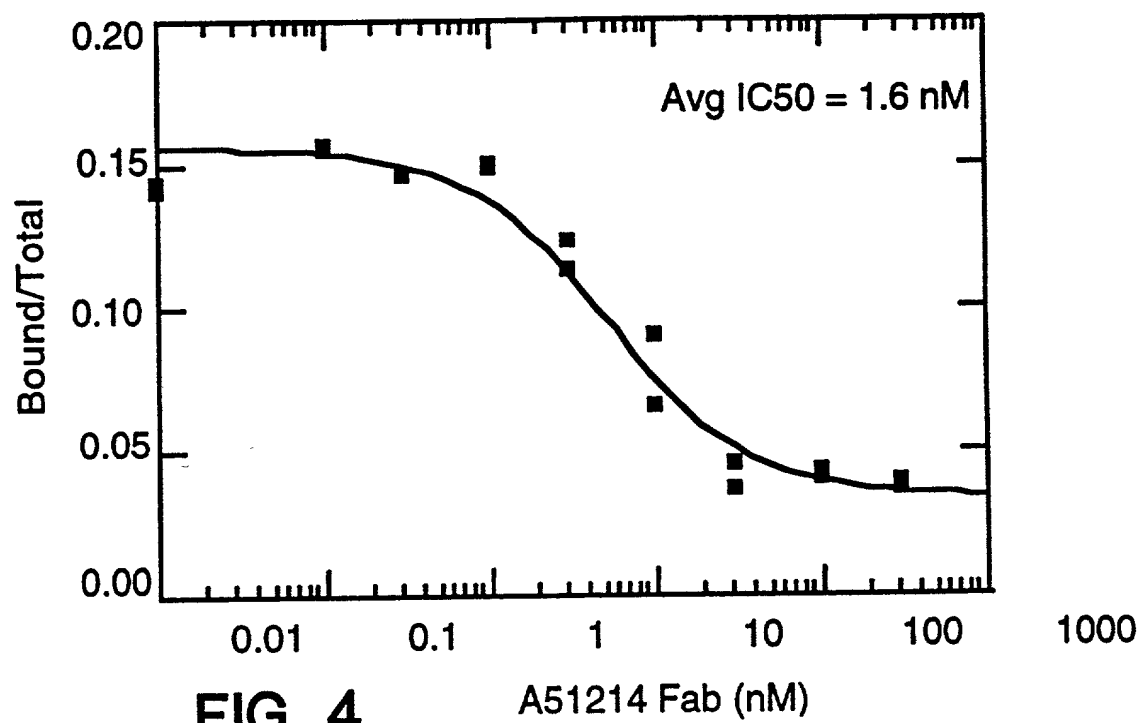
## Abstract of the Disclosure

5 Described are conjugates formed by an antibody fragment covalently attached to a non-proteinaceous polymer, wherein the apparent size of the conjugate is at least about 500 kD. The conjugates exhibit substantially improved half-life, mean residence time, and/or clearance rate in circulation as compared to the underivatized parental antibody fragment. Also described are conjugates directed against human vascular endothelial growth factor (VEGF), human p185  
10 receptor-like tyrosine kinase (HER2), human CD20, human CD18, human CD11a, human IgE, human apoptosis receptor-2 (Apo-2), human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), human tissue factor (TF), human  $\alpha 4\beta 7$  integrin, human GPIIb-IIIa integrin, human epidermal growth factor receptor (EGFR), human CD3, and human interleukin-2 receptor  $\alpha$ -chain (TAC) for diagnostic and therapeutic applications.

FIG. 1







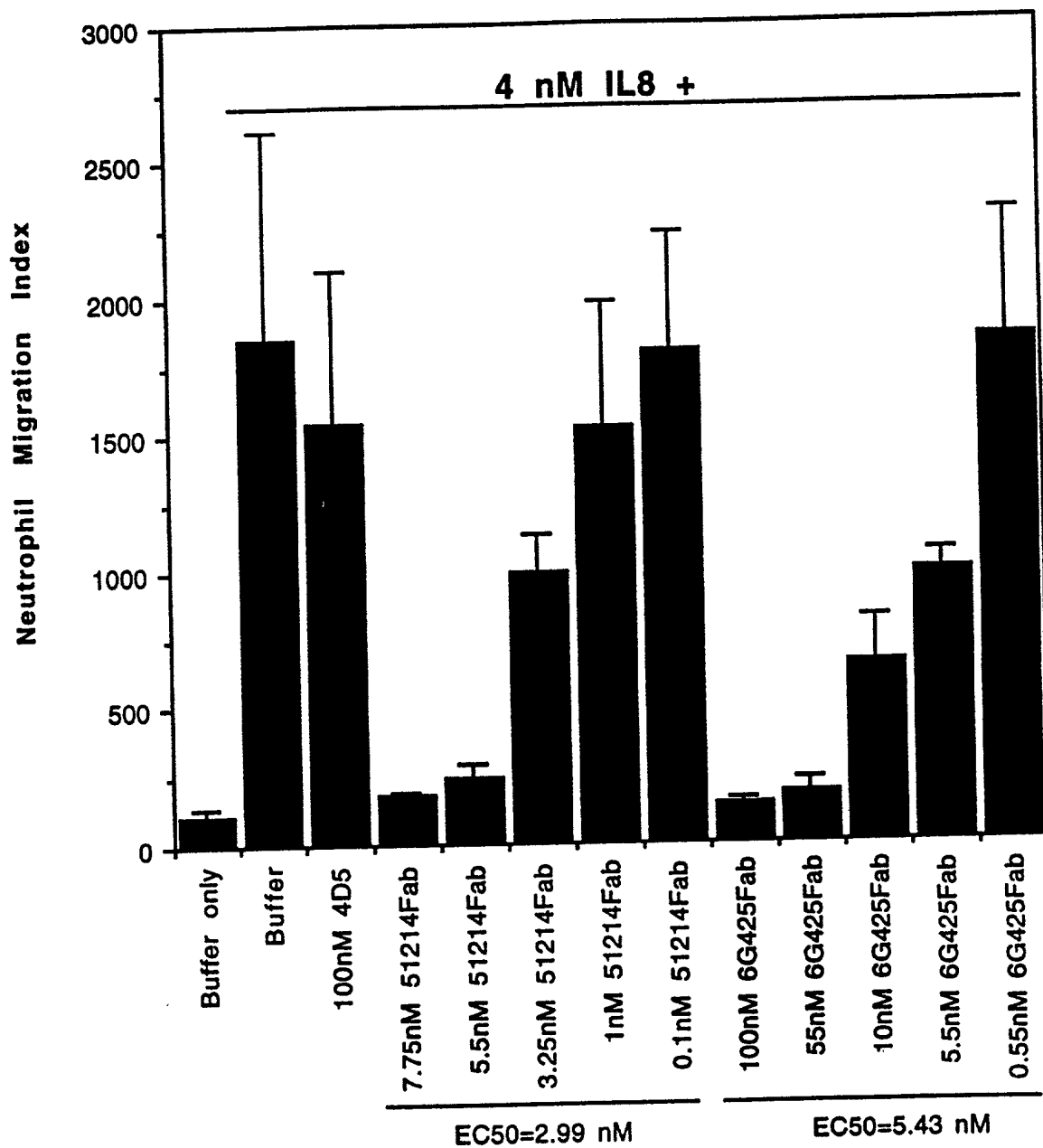


FIG. 6

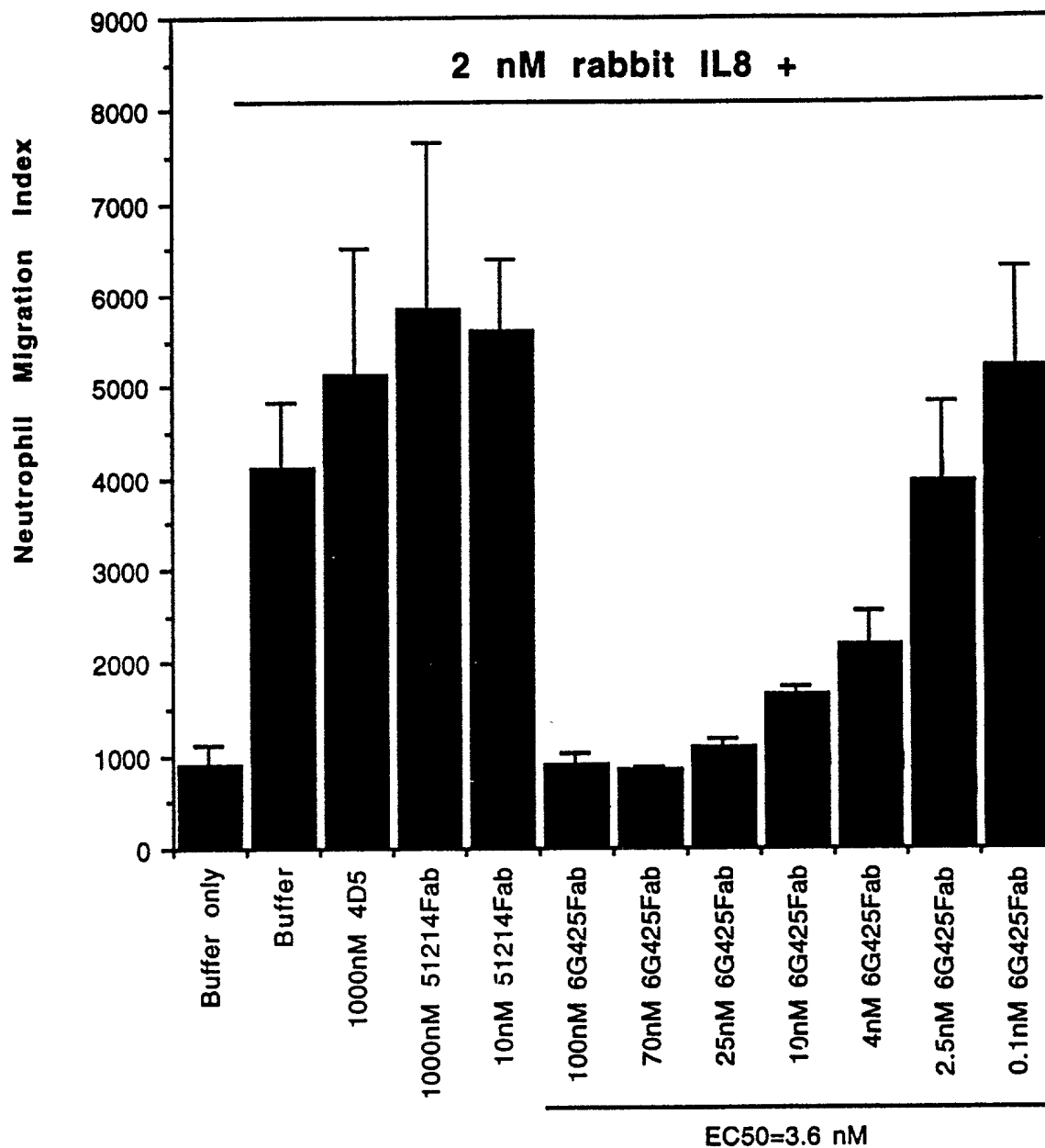


FIG. 7

FIG. 8

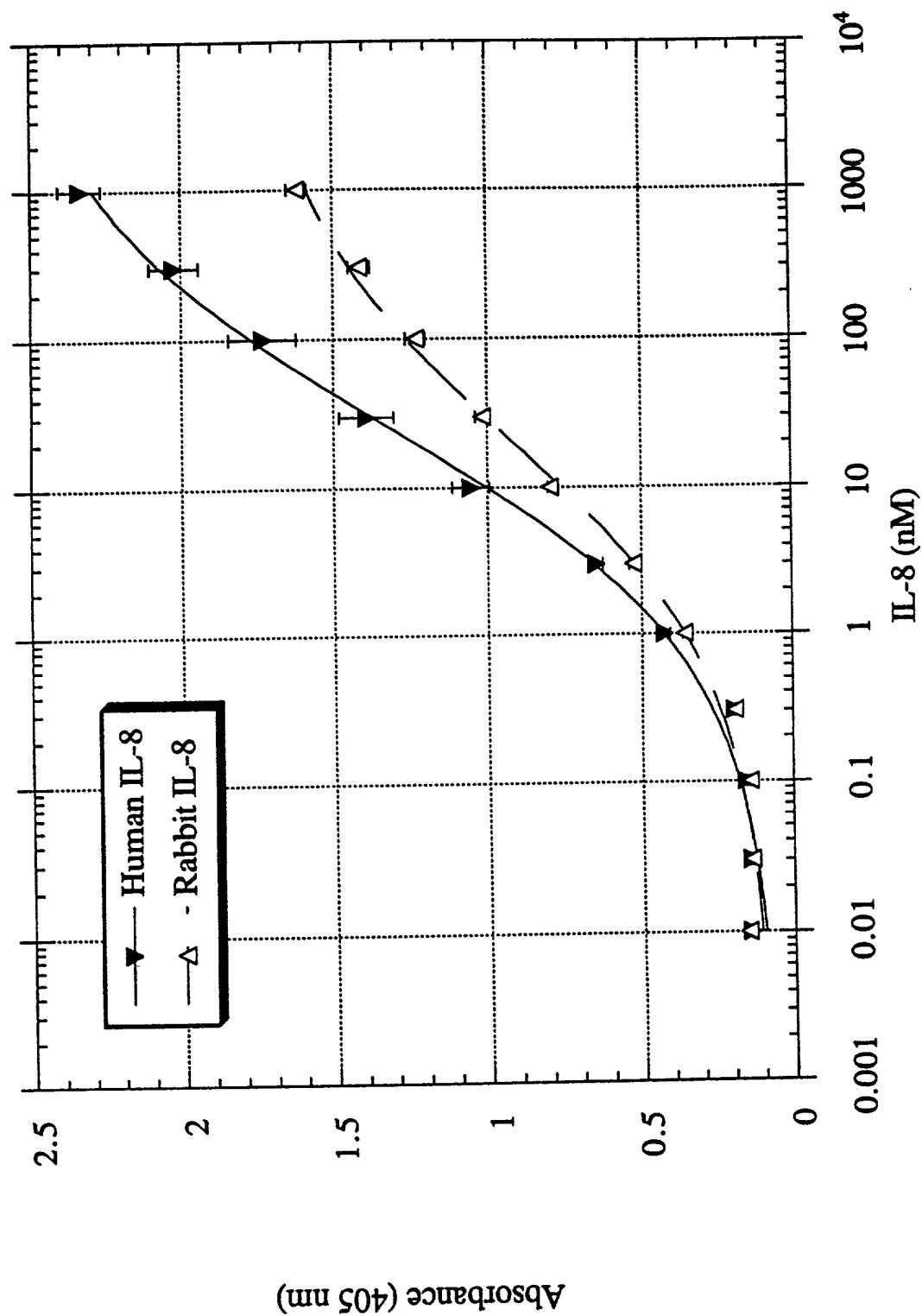
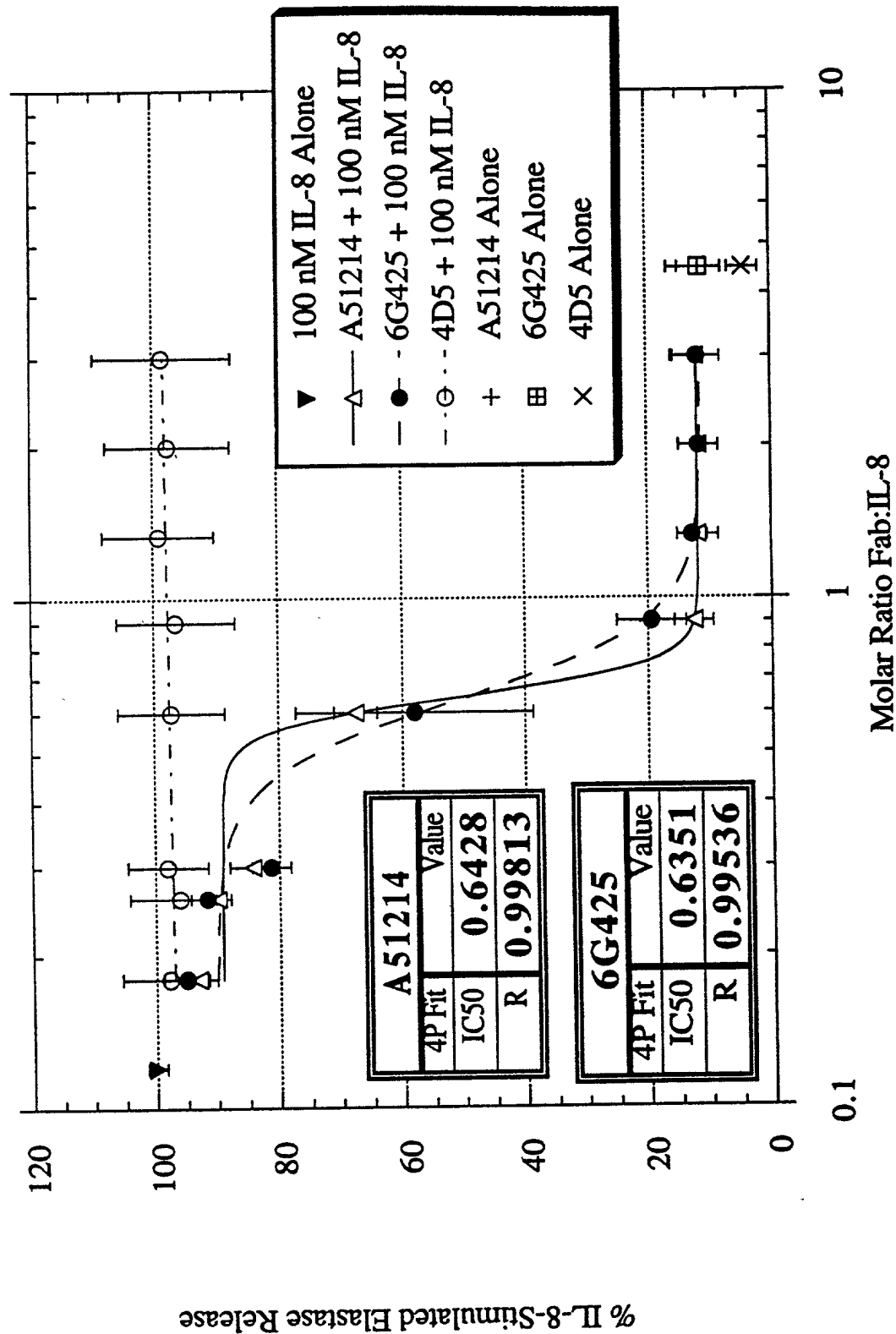


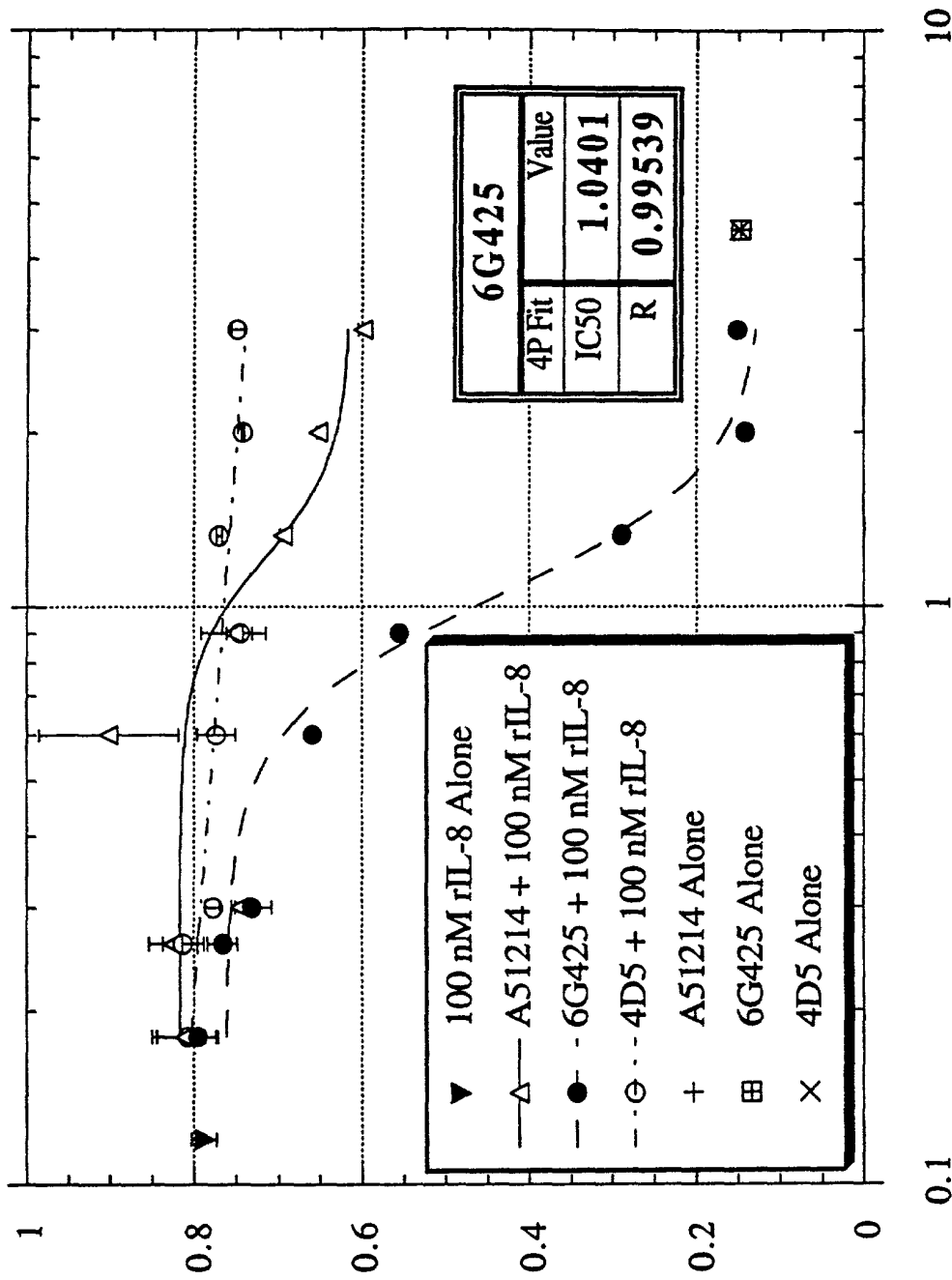
FIG. 9

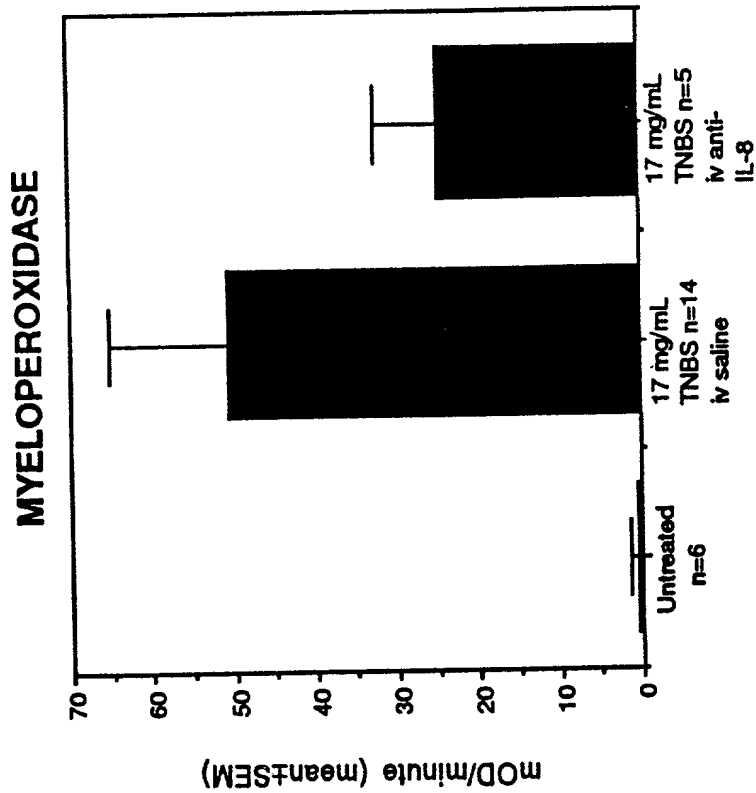




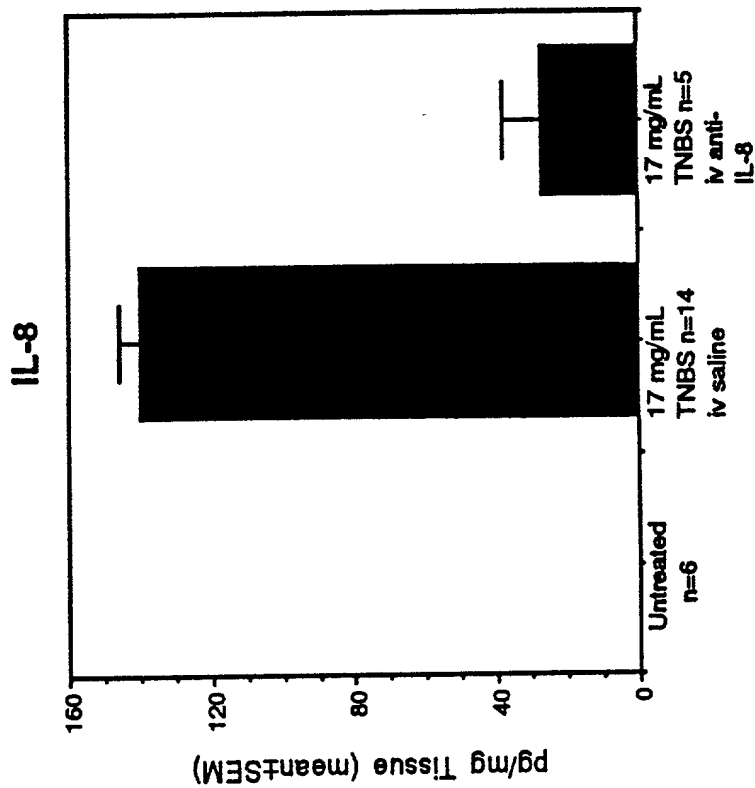
Absorbance (405 nm)

FIG. 10

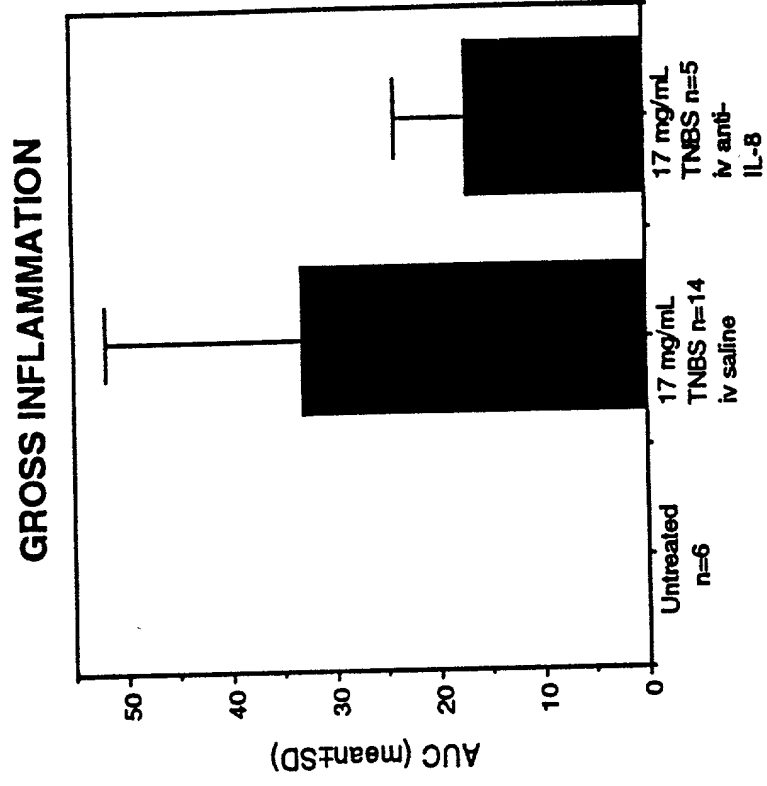




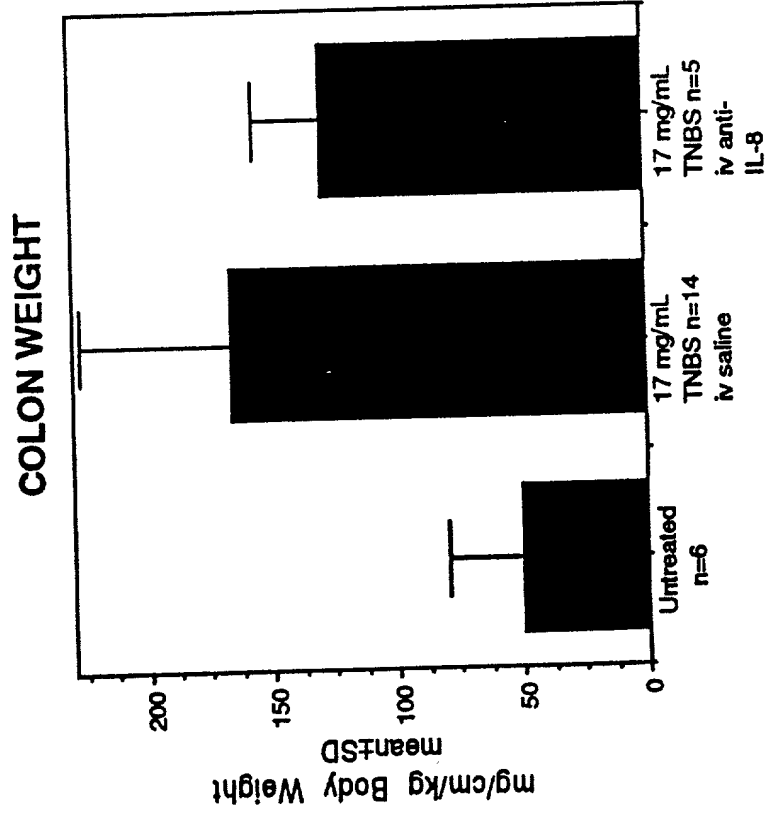
**FIG. 11A**



**FIG. 11B**



**FIG. 11D**



**FIG. 11C**

### EDEMA

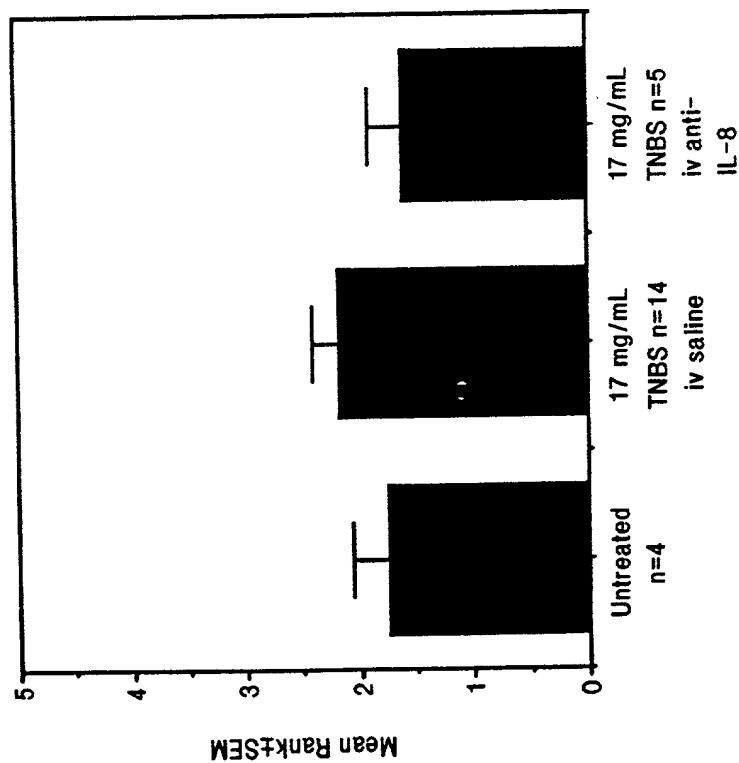


FIG. 11E

### EXTENT OF NECROSIS

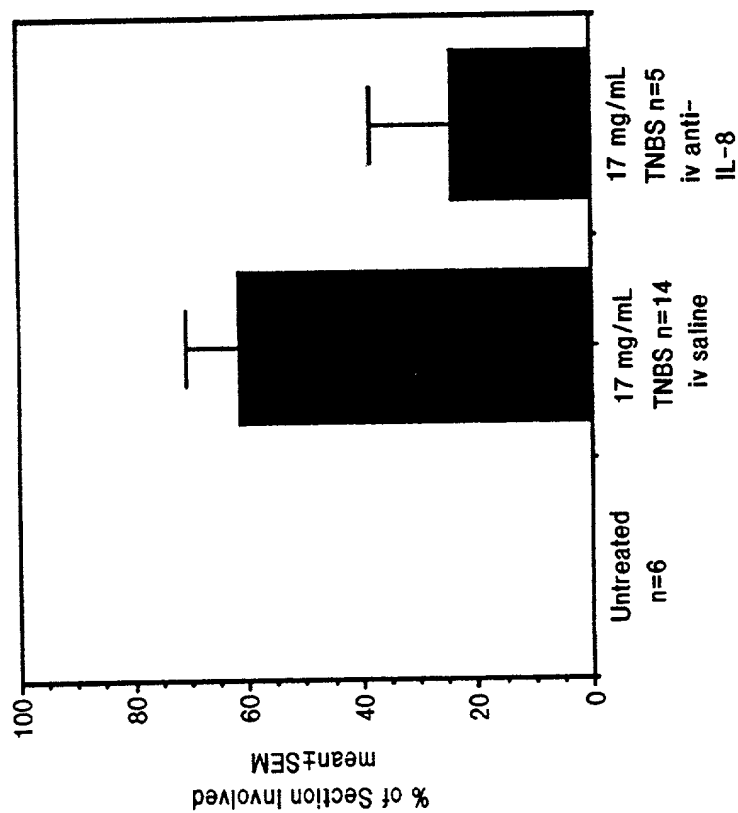
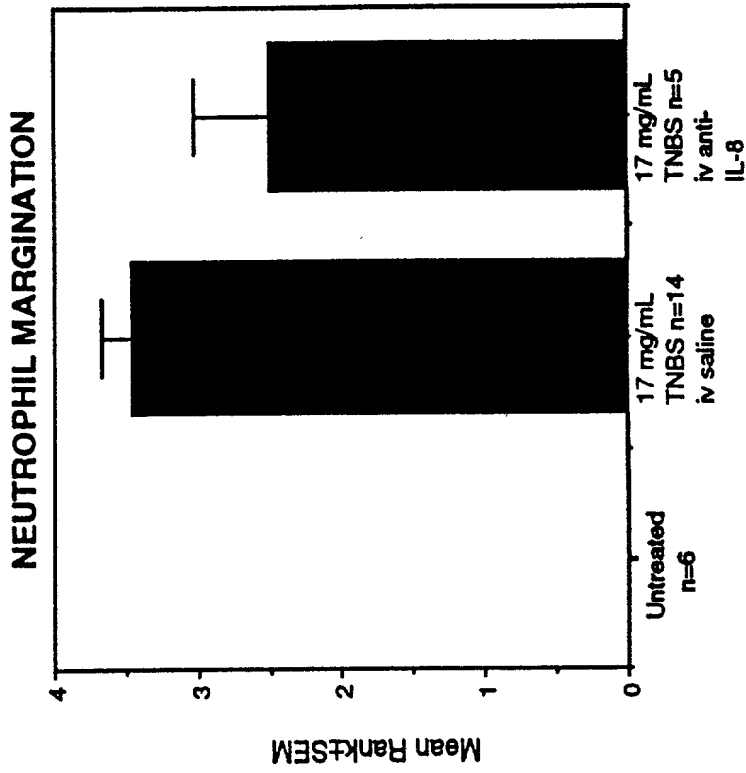
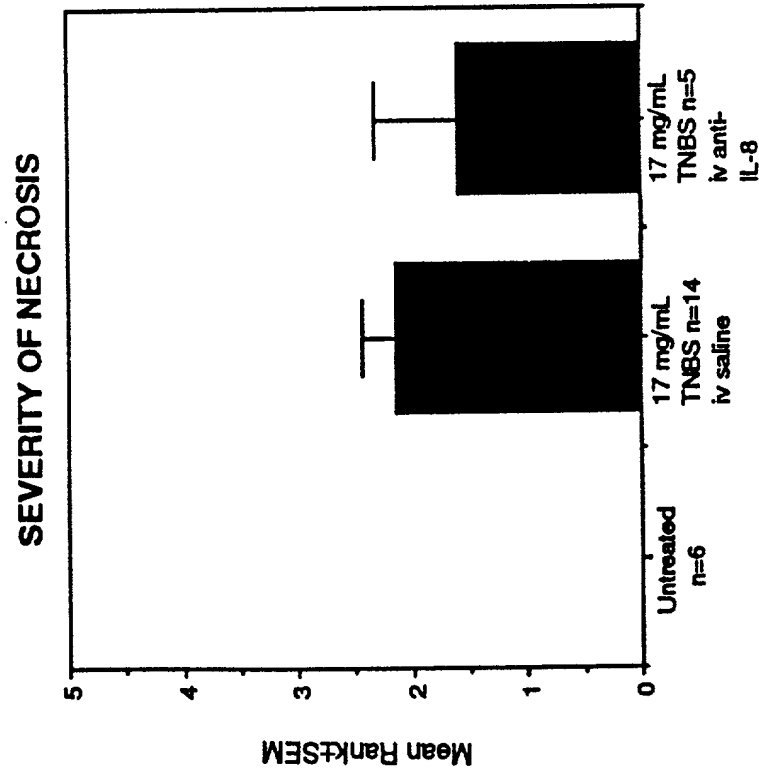


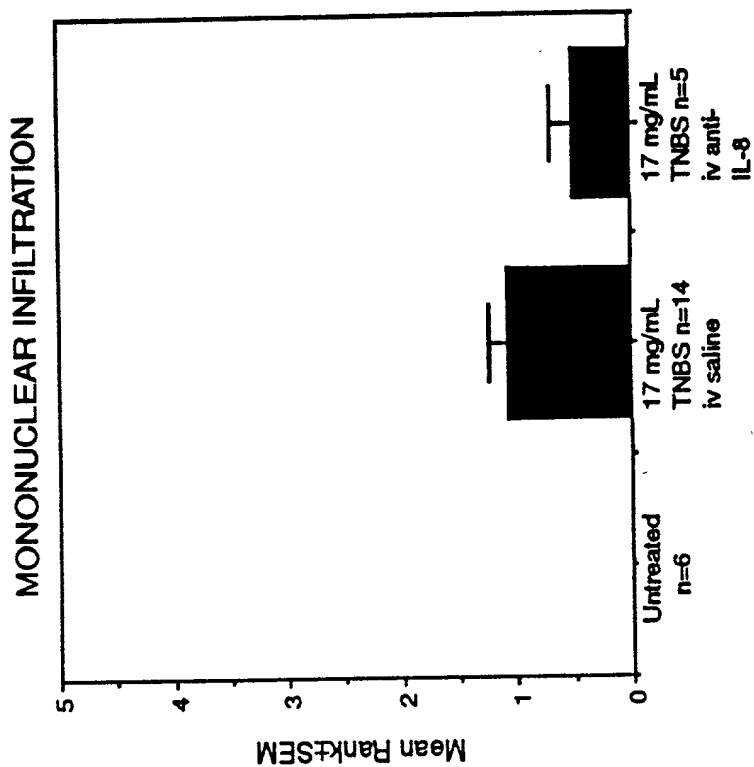
FIG. 11F



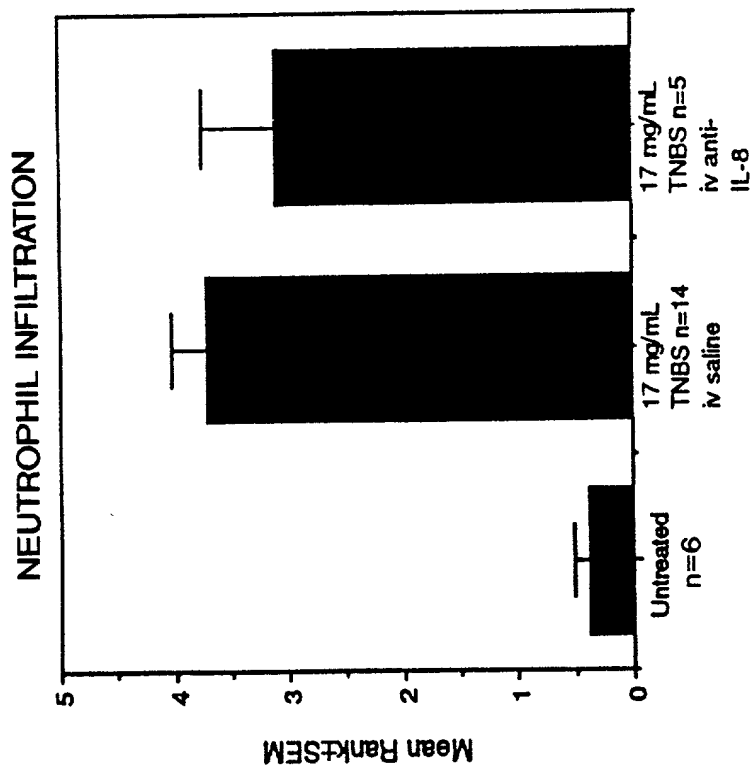
**FIG. 11H**



**FIG. 11G**

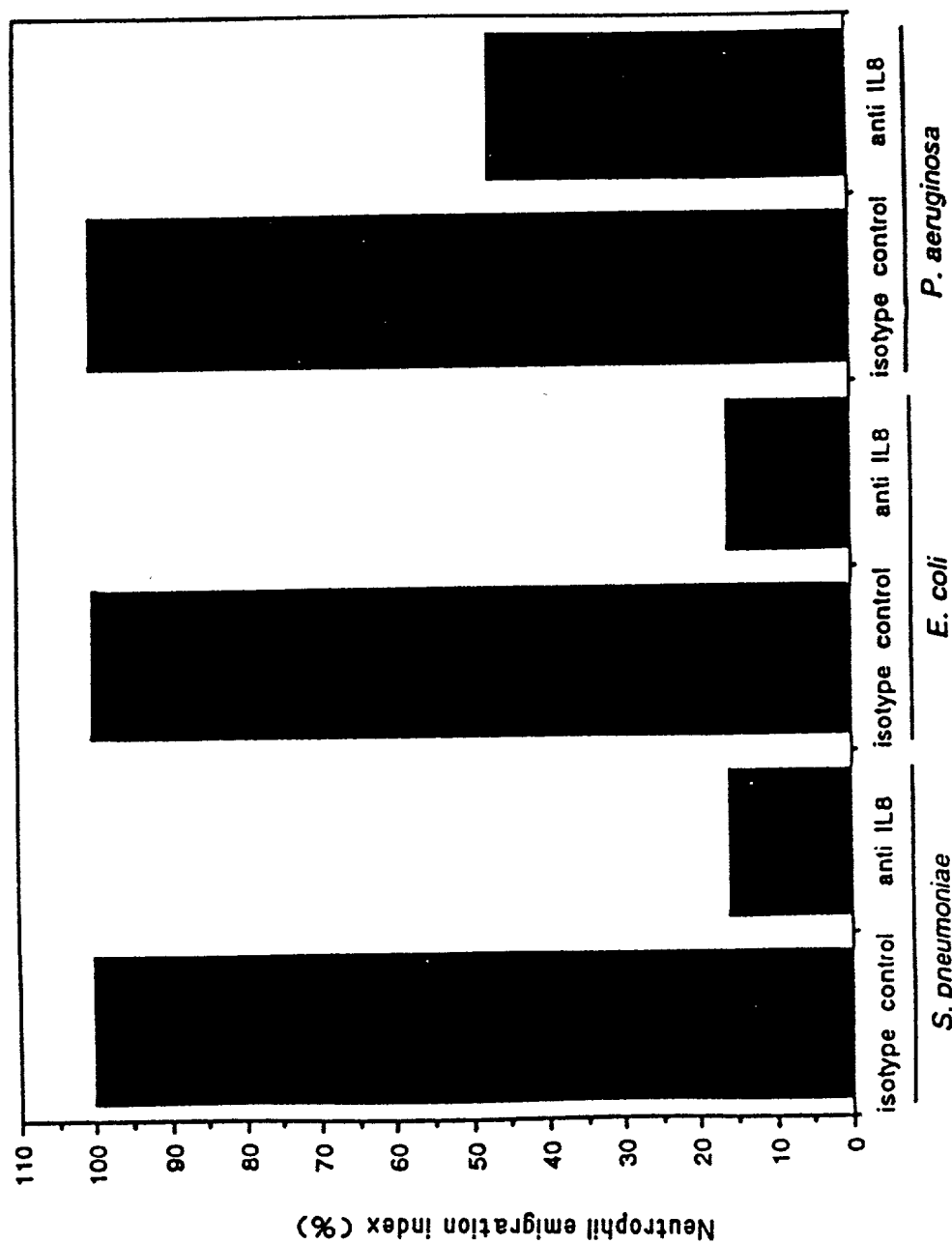


**FIG. 11J**



**FIG. 11I**

**FIG. 12**



Group (n=5 rabbits per group)

Light Chain Primers:

**FIG. 13**

MKLC-1, 22mer

5' CAGTCCAACCTGTTTCAGGACGCC 3'

MKLC-2, 22mer

5' GTGCTGCTCATGCTGTAGGTGC 3'

MKLC-3, 23mer

5' GAAGTTGATGTCTTGTGAGTGGC 3'

Heavy Chain Primers:

IGG2AC-1, 24mer

5' GCATCCTAGAGTCACCGAGGAGCC 3'

IGG2AC-2, 22mer

5' CACTGGCTCAGGGAAATAACCC 3'

IGG2AC-3, 22mer

5' GGAGAGCTGGGAAGGTGTGCAC 3'



## FIG. 14

Light chain forward primer

SL001A-2 35 mer

5' ACAAACGCGTACGCT GACATCGTCATGACCCAGTC 3'  
                                  T  T                  T  
  A

Light chain reverse primer

SL001B 37 mer

5' GCTCTTCGAATG GTGGGAAGATGGATACAGTTGGTGC 3'

Heavy chain forward primer

**FIG. 15**

SL002B 39 mer

5' CGATGGGCCCCGG ATAGACCGATGGGGCTGTTGTTTGGC 3'

T

C

G

A

Heavy chain reverse primer

SL002B 39-MER

5' CGATGGGCCCCGG ATAGACCGATGGGGCTGTTGTTTGGC 3'

T

A

G

1 GACATTGTCA TGACACAGTC TCAAAAATTC ATGTCCACAT CAGTAGGAGA CAGGGTCAGC  
 CTGTAACAGT ACTGTGTCAG AGTTTAAAG TACAGGTGTA GTCATCCTCT GTCCCAGTCG  
 1 D I V M T Q S Q K F M S T S V G D R V S  
 61 GTCACCTGCA AGGCCAGTCA GAATGTGGGT ACTAATGTAG CCTGGTATCA ACAGAAACCA  
 CAGTGGACGT TCCGGTCAGT CTTACACCCA TGATTACATC GGACCATAGT TGTCCTTTGGT  
 21 V T C K A S O N V G T N V A W Y Q Q K P  
 \* \* \* \* \*

CDR #1

121 GGGCAATCTC CTAAAGCACT GATTACTCG TCATCCTACC GGTACAGTGG AGTCCCTGAT  
 CCCGTTAGAG GATTTCGTGA CTAAATGAGC AGTAGGATGG CCATGTCACC TCAGGGACTA  
 41 G Q S P K A L I Y S S Y R Y S G V P D  
 \* \* \* \* \*

CDR #2

181 CGTTTCACAG GCAGTGGATC TGGACAGAT TTCACTCTCA CCATCAGCCA TGTGCAGTCT  
 GCGAAGTGTC CGTCACCTAG ACCCTGTCTA AAGTGAGAGT GGTAGTCGGT ACACGTCAGA  
 61 R F T G S G S G T D F T L T I S H V Q S  
 241 GAAGACTTGG CAGACTATTT CTGTCAGCAA TATAACATCT ATCCTCTCAC GTTCGGTCCT  
 CTTCTGAACC GTCTGATAAA GACAGTCGTT ATATTGTAGA TAGGAGAGTG CAAAGCCAGGA  
 81 E D L A D Y F C Q Q Y N I Y P L T F G P  
 \* \* \* \* \*

CDR #3

301 GGGACCAAGC TGGAGTTGAA ACGGGCTGAT GCTGCACCAC CAACTGTATC CATCTTCCCA  
 CCTTGGTTCG ACCTCAACTT TGCCCCGACTA CGACGTGGTG GTTGACATAG GTAGAAGGGT  
 101 G T K L E L K R A D A A P P T V S I F P

BstBI

361 CCAATTCGAA

GGTAAGCTT

121 P F E

FIG. 16

1 TTCTATTGCT ACAAACGCGT ACGCTGAGGT GCAGCTGGTG GAGTCTGGGG GAGGCTTAGT  
 AAGATAACGA TGTTTGCGCA TGC GACTCCA CGTCGACCAC CTCAGACCCC CTCCGAATCA  
 1 E V Q L V E S G G G L V

61 GCCGCCTGGA GGGTCCCTGA AACTCTCCTG TGCAGCCTCT GGATTCATAT TCAGTAGTTA  
 CGGCGGACCT CCCAGGGACT TTGAGAGGAC ACGTCGGAGA CCTAAGTATA AGTCATCAAT  
 13 P P G G S L K L S C A A S G F I F S S Y  
 \* \*

CDR #1

121 TGGCATGTCT TGGGTTCGCC AGACTCCAGG CAAGAGCCTG GAGTTGGTCG CAACCATTAA  
 ACCGTACAGA ACCCAAGCGG TCTGAGGTCC GTTCTCGGAC CTCAACCAGC GTTGGTAATT  
 33 G M S W V R Q T P G K S L E L V A T I N  
 \* \* \*

181 TAATAATGGT GATAGCACCT ATTATCCAGA CAGTGTGAAG GGCCGATTCA CCATCTCCCC  
 ATTATTACCA CTATCGTGGA TAATAGGTCT GTCACACTTC CCGGCTAAGT GGTAGAGGGC  
 53 N N G D S T Y Y P D S V K G R F T I S R  
 \* \* \* \* \* \* \* \* \* \*

CDR #2

241 AGACAATGCC AAGAACACCC TGTACCTGCA AATGAGCAGT CTGAAGTCTG AGGACACAGC  
 TCTGTTACGG TTCTTGTTGGG ACATGGACGT TTA CTGTCGTA GACTTCAGAC TCCTGTGTCG  
 73 D N A K N T L Y L Q M S S L K S E D T A

301 CATGTTTTAC TGTGCAAGAG CCCTCATTAG TTCGGCTACT TGGTTTGGTT ACTGGGGCCA  
 GTACAAAATG ACACGTTCTC GGGAGTAATC AAGCCGATGA ACCAAACCAA TGACCCCGGT  
 93 M F Y C A R A L I S S A T W F G Y W G Q  
 \* \* \* \* \* \* \* \* \*

CDR #3

361 AGGGACTCTG GTCACTGTCT CTGCAGCCAA AACAACAGCC CCATCTGTCT  
 TCCCTGAGAC CAGTGACAGA GACGTCGGTT TTGTTGTCGG GGTAGACAGA  
 113 G T L V T V S A A K T T A P S V Y

ApaI

411 ATCCGGG  
 TAGGCCC  
 130 P

FIG. 17

## FIG. 18

VL.front 31-MER

5' ACAAAACGCGTACGCTGATATCGTCATGACAG 3'  
VL.rear 31-MER

5' GCAGCATCAGCTCTTCGAAGCTCCAGCTTGG 3'

VH.front.SPE 21-MER

5' CCACTAGTACGCAAGTTCACG 3'

VH.rear 33-MER

5' GATGGGCCCCTTGGTGGAGGCTGCAGAGACAGTG 3'

1 ATGAAGAAGA ATATCGCATT TCTTCTTGCA TCTATGTTTCG TTTTCTCTAT TGCTACAAAC  
TACTTCTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG  
-23 M K K N I A F L L A S M F V F S I A T N

61 GCGTACGCTG ATATCGTCAT GACACAGTCT CAAAAATTCA TGTCCACATC AGTAGGAGAC  
CGCATGCGAC TATAGCAGTA CTGTGTCAGA GTTTTAAAGT ACAGGTGTAG TCATCCTCTG  
-3 A Y A D I V M T Q S Q K F M S T S V G D

121 AGGGTCAGCG TCACCTGCAA GGCCAGTCAG AATGTGGGTA CTAATGTAGC CTGGTATCAA  
TCCCAGTCGC AGTGGACGTT CCGGTCAGTC TTACACCCAT GATTACATCG GACCATAGTT  
18 R V S V T C K A S O N V G T N V A W Y Q  
\* \* \* \* \*

#### CDR #1

181 CAGAAACCAG GGCAATCTCC TAAAGCACTG ATTTACTCGT CATCCTACCG GTACAGTGGA  
GTCTTTGGTC CCGTTAGAGG ATTTCTGTGAC TAAATGAGCA GTAGGATGGC CATGTCACCT  
38 Q K P G Q S P K A L I Y S S S Y R Y S G  
\* \* \* \* \*

#### CDR #2

241 GTCCCTGATC GCTTCACAGG CAGTGGATCT GGGACAGATT TCACTCTCAC CATCAGCCAT  
CAGGGACTAG CGAAGTGTCC GTCACCTAGA CCCTGTCTAA AGTGAGAGTG GTAGTCGGTA  
58 V P D R F T G S G S G T D F T L T I S H

301 GTGCAGTCTG AAGACTTGGC AGACTATTTT TGTCAGCAAT ATAACATCTA TCCTCTCAGC  
CACGTCAGAC TTCTGAACCG TCTGATAAAG ACAGTCGTTA TATTGTAGAT AGGAGAGTGC  
78 V Q S E D L A D Y F C Q Q Y N I Y P L T  
\* \* \* \* \*

#### CDR #3

#### BstBI

361 TTCGGTCCTG GGACCAAGCT GGAGCTTCGA AGAGCTGTGG CTGCACCATC TGTCTTCATC  
AAGCCAGGAC CCTGGTTCGA CCTCGAAGCT TCTCGACACC GACGTGGTAG ACAGAAGTAG  
98 F G P G T K L E L R R A V A A P S V F I

421 TTCCCGCCAT CTGATGAGCA GTTGAAATCT GGAAGTGTCT CTGTTGTGTG CCTGCTGAAT  
AAGGGCGGTA GACTACTCGT CAACCTTTAGA CCTTGACGAA GACAACACAC GGACGACTTA  
118 F P P S D E Q L K S G T A S V V C L L N

481 AACTTCTATC CCAGAGAGGC CAAAGTACAG TGGAAGGTGG ATAACGCCCT CCAATCGGGT  
TTGAAGATAG GGTCTCTCCG GTTTCATGTC ACCTTCCACC TATTGCGGGA GGTTAGCCCA  
138 N F Y P R E A K V Q W K V D N A L Q S G

541 AACTCCCAGG AGAGTGTAC AGAGCAGGAC AGCAAGGACA GCACCTACAG CCTCAGCAGC  
TTGAGGGTCC TCTCACAGTG TCTCGTCCTG TCGTTCCTGT CGTGGATGTC GGAGTCGTCTG  
158 N S Q E S V T E Q D S K D S T Y S L S S

601 ACCCTGACGC TGAGCAAAGC AGACTACGAG AAACACAAAG TCTACGCCTG CGAAGTCACC  
TGGGACTGCG ACTCGTTTCG TCTGATGCTC TTTGTGTTTC AGATGCGGAC GCTTCAGTGG  
178 T L T L S K A D Y E K H K V Y A C E V T

661 CATCAGGGCC TGAGCTCGCC CGTCACAAAG AGCTTCAACA GGGGAGAGTG  
GTAGTCCCGG ACTCGAGCGG GCAGTGTTTC TCGAAGTTGT CCCCTCTCAC  
198 H Q G L S S P V T K S F N R G E C

711 TTAA

AATT

216 O

FIG. 19

1 ATGAAAAAGA ATATCGCATT TCTTCTTGCA TCTATGTTTCG TTTTCTCTAT TGCTACAAAC  
 TACTTTTTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG  
 -23 M K K N I A F L L A S M F V F S I A T N  
 61 GCGTACGCTG AGGTGCAGCT GGTGGAGTCT GGGGGAGGCT TAGTGCCGCC TGGAGGGTCC  
 CGCATGCGAC TCCACGTCGA CCACCTCAGA CCCCTCCGA ATCACGGCGG ACCTCCCAGG  
 -3 A Y A E V Q L V E S G G G L V P P G G S  
 121 CTGAAACTCT CCTGTGCAGC CTCTGGATTCT ATATTCAGTA GTTATGGCAT GTCTTGGGTT  
 GACTTTGAGA GGACACGTCG GAGACCTAAG TATAAGTCAT CAATACCGTA CAGAACCCAA  
 18 L K L S C A A S G F I F S S Y G M S W V  
 \* \* \* \* \*

#### CDR #1

181 CGCCAGACTC CAGGCAAGAG CCTGGAGTTG GTCGCAACCA TTAATAATAA TGGTGATAGC  
 GCGGTCTGAG GTCCGTTCTC GGACCTCAAC CAGCGTTGGT AATTATTATT ACCACTATCG  
 38 R Q T P G K S L E L V A T I N N N G D S  
 \* \* \* \* \*

241 ACCTATTATC CAGACAGTGT GAAGGGCCGA TTCACCATCT CCCGAGACAA TGCCAAGAAC  
 TGGATAATAG GTCTGTCACA CTTCCCGGCT AAGTGGTAGA GGGCTCTGTT ACGGTTCTTG  
 58 T Y Y P D S V K G R F T I S R D N A K N  
 \* \* \* \* \*

#### CDR #2

301 ACCCTGTACC TGCAAATGAG CAGTCTGAAG TCTGAGGACA CAGCCATGTT TTACTGTGCA  
 TGGGACATGG ACGTTTACTC GTCAGACTTC AGACTCCTGT GTCGGTACAA AATGACACGT  
 78 T L Y L Q M S S L K S E D T A M F Y C A  
 361 AGAGCCCTCA TTAGTTCGGC TACTTGTTTT GGTACTGGG GCCAAGGGAC TCTGGTCACT  
 TCTCGGGAGT AATCAAGCCG ATGAACCAAA CCAATGACCC CGGTTCCCTG AGACCAGTGA  
 98 R A L I S S A T W F G Y W G Q G T L V T  
 \* \* \* \* \*

#### CDR #3

ApaI

421 GTCTCTGCAG CCTCCACCAA GGGCCCATCG GTCTTCCCC TGGCACCCCTC CTCCAAGAGC  
 CAGAGACGTC GGAGGTGGTT CCCGGGTAGC CAGAAGGGGG ACCGTGGGAG GAGGTTCTCG  
 118 V S A A S T K G P S V F P L A P S S K S  
 481 ACCTCTGGGG GCACAGCGGC CCTGGGCTGC CTGGTCAAGG ACTACTTCCC CGAACCGGTG  
 TGGAGACCCC CGTGTGCGCCG GGACCCGACG GACCAGTTCC TGATGAAGGG GCTTGGCCAC  
 138 T S G G T A A L G C L V K D Y F P E P V  
 541 ACGGTGTCGT GGAATCAGG CGCCCTGACC AGCGGCGTGC ACACCTTCCC GGCTGTCCTA  
 TGCCACAGCA CCTTGAGTCC GCGGGACTGG TCGCCGCACG TGTGGAAGGG CCGACAGGAT  
 158 T V S W N S G A L T S G V H T F P A V L  
 601 CAGTCCTCAG GACTCTACTC CCTCAGCAGC GTGGTGACCG TGCCCTCCAG CAGCTTGGGC  
 GTCAGGAGTC CTGAGATGAG GGAGTCGTCG CACCACTGGC ACGGGAGGTC GTCGAACCCG  
 178 Q S S G L Y S L S S V V T V P S S S L G

FIG. 20A

661 ACCCAGACCT ACATCTGCAA CGTGAATCAC AAGCCCAGCA ACACCAAGGT GGACAAGAAA  
TGGGTCTGGA TGTAGACGTT GCACTTAGTG TTCGGGTCGT TGTGGTTCCA CCTGTTCTTT  
198 T Q T Y I C N V N H K P S N T K V D K K  
721 GTTGAGCCCA AATCTTGTGA CAAAACTCAC ACATGA  
CAACTCGGGT TTAGAACACT GTTTTGAGTG TGTA  
218 V E P K S C D K T H T O

FIG. 20B



Light Chain Primers:

MKLC-1, 22mer

5' CAGTCCAACGTGTCAGGACGCC 3'

MKLC-2, 22mer

5' GTGCTGCTCATGCTGTAGGTGC 3'

MKLC-3, 23mer

5' GAAGTTGATGTCTTGTGAGTGGC 3'

Heavy Chain Primers:

IGG2AC-1, 24mer

5' GCATCCTAGAGTCACCGAGGAGCC 3'

IGG2AC-2, 22mer

5' CACTGGCTCAGGGAAATAACCC 3'

IGG2AC-3, 22mer

5' GGAGAGCTGGGAAGGTGTGCAC 3'

**FIG. 21**

Light chain forward primer

6G4.light.Nsi 36-MER

5' CCAATGCATACGCT GAC ATC GTG ATG ACC CAG ACC CC 3'  
                          T    T                  T          T  
  A          A

Light chain reverse primer

6G4.light.Mun 35-MER

5' AGA TGT CAA TTG CTC ACT GGA TGG TGG GAA GAT GG 3'

**FIG. 22**

Heavy chain forward primer

6G4.heavy.Mlu 32-MER

5' CAAACGCGTACGCT GAG ATC CAG CTG CAG CAG 3'  
                                  T                  C

Heavy chain reverse primer

SL002B 39-MER

5' CGATGGGCCCGG ATAGACCGATGGGGCTGTTGTTTTGGC 3'  
                                  T  
                                  A  
                                  G

**FIG. 23**

70 G ATATCGTGAT GACACAGACA CCACTCTCCC TGCCTGTCAG TCTTGGAGAT  
 C TATAGCACTA CTGTGTCTGT GGTGAGAGGG ACGGACAGTC AGAACCTCTA  
 1 D I V M T Q T P L S L P V S L G D

121 CAGGCCTCCA TCTCTTGCAG ATCTAGTCAG AGCCTTGTAC ACGGTATTGG AAACACCTAT  
 GTCCGGAGGT AGAGAACGTC TAGATCAGTC TCGGAACATG TGCCATAACC TTTGTGGATA  
 18 Q A S I S C R S S O S L V H G I G N T Y  
 \* \* \* \* \* \* \* \* \* \* \* \* \*

CDR #1

181 TTACATTGGT ACCTGCAGAA GCCAGGCCAG TCTCCAAAGC TCCTGATCTA CAAAGTTTCC  
 AATGTAACCA TGGACGTCTT CGGTCCGGTC AGAGGTTTCG AGGACTAGAT GTTTCAAAGG  
 38 L H W Y L Q K P G Q S P K L L I Y K V S  
 \* \* \* \* \* \* \* \*

CDR #2

241 AACCGATTTT CTGGGGTCCC AGACAGGTTC AGTGGCAGTG GATCAGGGAC AGATTTTACA  
 TTGGCTAAAA GACCCAGGG TCTGTCCAAG TCACCGTCAC CTAGTCCCTG TCTAAAGTGT  
 58 N R F S G V P D R F S G S G S G T D F T  
 \* \* \* \* \*

301 CTCAGGATCA GCAGAGTGGA GGCTGAGGAT CTGGGACTTT ATTTCTGCTC TCAAAGTACA  
 GAGTCCTAGT CGTCTCACCT CCGACTCCTA GACCCTGAAA TAAAGACGAG AGTTTCATGT  
 78 L R I S R V E A E D L G L Y F C S Q S T  
 \* \* \* \* \*

CDR #3

361 CATGTTCCGC TCACGTTCGG TGCTGGGACC AAGCTGGAGC TGAAACGGGC TGATGCTGCA  
 GTACAAGGCG AGTGCAAGCC ACGACCCTGG TTCGACCTCG ACTTTGCCCCG ACTACGACGT  
 98 H V P L T F G A G T K L E L K R A D A A  
 \* \* \* \* \*

MunI

421 CCAACTGTAT CCATCTTCCC ACCATCCAGT GAGCAATTGA  
 GGTTGACATA GGTAGAAGGG TGGTAGGTCA CTCGTTAACT  
 118 P T V S I F P P S S E Q L K

FIG. 24

70 G AGATTCAGCT GCAGCAGTCT GGACCTGAGC TGATGAAGCC TGGGGCTTCA  
 C TCTAAGTCGA CGTCGTCAGA CCTGGACTCG ACTACTTCGG ACCCCGAAGT  
 1 E I Q L Q Q S G P E L M K P G A S

121 GTGAAGATAT CCTGCAAGGC TTCTGGTTAT TCATTAGTA GCCACTACAT GCACTGGGTG  
 CACTTCTATA GGACGTTCCG AAGACCAATA AGTAAGTCAT CGGTGATGTA CGTGACCCAC  
 18 V K I S C K A S G Y S F S S H Y M H W V  
 \* \* \* \* \*

CDR #1

181 AAGCAGAGCC ATGGAAAGAG CCTTGAGTGG ATTGGCTACA TTGATCCTTC CAATGGTGAA  
 TTCGTCTCGG TACCTTTCTC GGAAGTCACC TAACCGATGT AACTAGGAAG GTTACCACTT  
 38 K Q S H G K S L E W I G Y I D P S N G E  
 \* \* \* \* \*

CDR #2

241 ACTACTTACA ACCAGAAATT CAAGGGCAAG GCCACATTGA CTGTAGACAC ATCTTCCAGC  
 TGATGAATGT TGGTCTTTAA GTTCCCGTTC CGGTGTAAGT GACATCTGTG TAGAAGGTCG  
 58 T T Y N Q K F K G K A T L T V D T S S S  
 \* \* \* \* \*

301 ACAGCCAACG TGCATCTCAG CAGCCTGACA TCTGATGACT CTGCAGTCTA TTTCTGTGCA  
 TGTCGGTTGC ACGTAGAGTC GTCGGACTGT AGACTACTGA GACGTCAGAT AAAGACACGT  
 78 T A N V H L S S L T S D D S A V Y F C A

361 AGAGGGGACT ATAGATACAA CGGCGACTGG TTTTTCGATG TCTGGGGCGC AGGGACCACG  
 TCTCCCCTGA TATCTATGTT GCCGCTGACC AAAAAGCTAC AGACCCCGCG TCCCTGGTGC  
 98 R G D Y R Y N G D W F F D V W G A G T T  
 \* \* \* \* \*

CDR #3

BstEII ApaI  
 421 GTCACCGTCT CCTCCGCCAA AACCGACAGC CCCATCGGTC TATCCGGGCC  
 CAGTGGCAGA GGAGGCGGAT TTGGCTGTCTG GGGTAGCCAG ATAGGCCCCG  
 118 V T V S S A K T D S P I G L S G P

471 CATC

GTAG

135 I

FIG. 25

5' CTTGGTGGAGGCGGAGGAGACG 3'

Mutagenesis Primer for 6G425VL

DS/VF 38MER

5' GAAACGGGCTGTTGCTGCACCAACTGTATTCATCTTCC 3'

SYN.BstEII 31 MER

5' GTCACCGTCT CCTCCGCCTC CACCAAGGGC C 3'

SYN.Apa 22 MER

5' CTTGGTGGAGGCGGAGGAGACG 3'

**FIG. 26**

1 ATGAAGAAGA ATATCGCATT TCTTCTTGCA TCTATGTTTCG TTTTCTCTAT TGCTACAAAT  
 TACTTCTTCT TATAGCGTAA AGAAGAACGT AGATAACAAGC AAAAAAGATA ACGATGTTTA  
 -23 M K K N I A F L L A S M F V F S I A T N

61 GCATACGCTG ATATCGTGAT GACACAGACA CCACTCTCCC TGCTGTCTAG TCTTGAGAT  
 CGTATGCGAC TATAGCACTA CTGTGTCTGT GGTGAGAGGG ACGGACAGTC AGAACCTCTA  
 -3 A Y A D I V M T Q T P L S L P V S L G D

121 CAGGCCTCCA TCTCTTGCAAG ATCTAGTCAG AGCCTTGTAC ACGGTATTGG AAACACCTAT  
 GTCCGGAGGT AGAGAACGTC TAGATCAGTC TCGGAACATG TGCCATAACC TTTGTGGATA  
 18 Q A S I S C R S S O S L V H G I G N T Y  
 \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \*

CDR #1

181 TTACATTGGT ACCTGCAGAA GCCAGGCCAG TCTCCAAAGC TCCTGATCTA CAAAGTTTCC  
 AATGTAACCA TGGACGTCTT CGGTCCGGTC AGAGGTTTTCG AGGACTAGAT GTTTCAAAGG  
 38 L H W Y L Q K P G Q S P K L L I Y K V S  
 \* \* \* \* \* \* \* \* \* \*

CDR #2

241 AACCGATTTT CTGGGGTCCC AGACAGGTTT AGTGGCAGTG GATCAGGGAC AGATTTTACA  
 TTGGCTAAAA GACCCAGGG TCTGTCCAAG TCACCGTCAC CTAGTCCCTG TCTAAAGTGT  
 58 N R F S G V P D R F S G S G S G T D F T  
 \* \* \* \* \*

301 CTCAGGATCA GCAGAGTGGA GGCTGAGGAT CTGGGACTTT ATTTCTGCTC TCAAAGTACA  
 GAGTCCTAGT CGTCTCACCT CCGACTCCTA GACCCTGAAA TAAAGACGAG AGTTTCATGT  
 78 L R I S R V E A E D L G L Y F C S Q S T  
 \* \* \* \* \*

CDR #3

361 CATGTTCCGC TCACGTTTCGG TGCTGGGACC AAGCTGGAGC TGAAACGGGC TGTGCTGCA  
 GTACAAGGCG AGTGCAAGCC ACGACCCTGG TTCGACCTCG ACTTTGCCCCG ACAACGACGT  
 98 H V P L T F G A G T K L E L K R A V A A  
 \* \* \* \* \*

421 CCAACTGTAT TCATCTTCCC ACCATCCAGT GAGCAATTGA AATCTGGAAC TGCCTCTGTT  
 GGTGACATA AGTAGAAGGG TGGTAGGTCA CTCGTAACT TTAGACCTTG ACGGAGACAA  
 118 P T V F I F P P S S E Q L K S G T A S V

481 GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA GGTGGATAAC  
 CACACGGACG ACTTATTGAA GATAGGGTCT CTCCGGTTTC ATGTCACCTT CCACCTATTG  
 138 V C L L N N F Y P R E A K V Q W K V D N

541 GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC AGGACAGCAA GGACAGCACC  
 CGGGAGGTTA GCGCATGAG GGTCTCTCA CAGTGTCTCG TCCTGTCTGT CCTGTCTGTG  
 158 A L Q S G N S Q E S V T E Q D S K D S T

601 TACAGCCTCA GCAGCACCCT GACGCTGAGC AAAGCAGACT ACGAGAAACA CAAAGTCTAC  
 ATGTCGGAGT CGTCGTGGGA CTGCGACTCG TTTCGTCTGA TGCTCTTTGT GTTTCAGATG  
 178 Y S L S S T L T L S K A D Y E K H K V Y

FIG. 27A

CGCTGCGAAG TCACCCATCA GGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA

661 GCCTGCGAAG TCACCCATCA GGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA  
CGGACGCTTC AGTGGGTAGT CCGGACTCG AGCGGCAGT GTTCTCGAA GTTGTCCCCT  
198 A C E V T H Q G L S S P V T K S F N R G

721 GAGTGTAA  
CTCACAATT  
218 E C O

FIG. 27B



1 ATGAAAAGA ATATCGCATT TCTTCTTGCA TCTATGTTTCG TTTTCTCTAT TGCTACAAAC  
TACTTTTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG  
-23 M K K N I A F L L A S M F V F S I A T N

61 GCGTACGCTG AGATTTCAGCT GCAGCAGTCT GGACCTGAGC TGATGAAGCC TGGGGCTTCA  
CGCATGCGAC TCTAAGTCGA CGTCGTCAGA CCTGGACTCG ACTACTTCGG ACCCCGAAGT  
-3 A Y A E I Q L Q Q S G P E L M K P G A S

121 GTGAAGATAT CCTGCAAGGC TTCTGGTTAT TCATTTCAGTA GCCACTACAT GCACTGGGTG  
CACTTCTATA GGACGTTCCG AAGACCAATA AGTAAGTCAT CGGTGATGTA CGTGACCCAC  
18 V K I S C K A S G Y S F S S H Y M H W V

\* \* \* \*

CDR #1

181 AAGCAGAGCC ATGGAAAGAG CCTTGAGTGG ATTGGCTACA TTGATCCTTC CAATGGTGAA  
TTCGTCTCGG TACCTTTCTC GGAAGTCACC TAACCGATGT AACTAGGAAG GTTACCACTT  
38 K Q S H G K S L E W I G Y I D P S N G E

\* \* \* \* \*

CDR #2

241 ACTACTTACA ACCAGAAATT CAAGGGCAAG GCCACATTGA CTGTAGACAC ATCTTCCAGC  
TGATGAATGT TGGTCTTTAA GTTCCCGTTC CGGTGTAAGT GACATCTGTG TAGAAGGTGC  
58 T T Y N Q K F K G K A T L T V D T S S S  
\* \* \* \* \*

301 ACAGCCAACG TGCATCTCAG CAGCCTGACA TCTGATGACT CTGCAGTCTA TTTCTGTGCA  
TGTCGGTTGC ACGTAGAGTC GTCGGACTGT AGACTACTGA GACGTCAGAT AAAGACACGT  
78 T A N V H L S S L T S D D S A V Y F C A

361 AGAGGGGACT ATAGATACAA CGGCGACTGG TTTTTCGATG TCTGGGGCGC AGGGACCACG  
TCTCCCTGA TATCTATGTT GCCGCTGACC AAAAAAGCTAC AGACCCCGCG TCCCTGGTGC  
98 R G D Y R Y N G D W F F D V W G A G T T  
\* \* \* \* \*

CDR #3

421 GTCACCGTCT CCTCCGCCTC CACCAAGGGC CCATCGGTCT TCCCCCTGGC ACCCTCCTCC  
CAGTGGCAGA GGAGGCGGAG GTGGTTCCCG GGTAGCCAGA AGGGGGACCG TGGGAGGAGG  
118 V T V S S A S T K G P S V F P L A P S S

481 AAGAGCACCT CTGGGGGCAC AGCGGCCCTG GGCTGCCTGG TCAAGGACTA CTTCCCCGAA  
TTCTCGTGGA GACCCCGTG TCGCCGGGAC CCGACGGACC AGTTCCTGAT GAAGGGGCTT  
138 K S T S G G T A A L G C L V K D Y F P E

541 CCGGTGACGG TGTCGTGGAA CTCAGGCGCC CTGACCAGCG GCGTGCACAC CTTCCCGGCT  
GGCCACTGCC ACAGCACCTT GAGTCCGCGG GACTGGTCGC CGCACGTGTG GAAGGGCCGA  
158 P V T V S W N S G A L T S G V H T F P A

601 GTCCTACAGT CCTCAGGACT CTACTCCCTC AGCAGCGTGG TGACCGTGCC CTCCAGCAGC  
CAGGATGTCA GGAGTCCTGA GATGAGGGAG TCGTCGCACC ACTGGCACGG GAGGTCGTGC  
178 V L Q S S G L Y S L S S V V T V P S S S

FIG. 28A



## Variable Light Chain Domain

	10	20	abcde	30	40
<b>6G425</b>	DIVMTQTPLSLPVS LGDQASISCRSSQSLVHGIGNTYLHWYLQKPGQSPKLLIY				
	#	#	#	#	#
<b>F(ab)-1</b>	DIQMTQSPSSLSASVGRVTITCRSSQSLVHGIGNTYLHWYQQKPGKAPKLLIY				
	#				
<b>humkI</b>	DIQMTQSPSSLSASVGRVTITCRASKTI-----SKYLAWYQQKPGKAPKLLIY				
	=====				
	+++++				
	<b>L1</b>				

	50	60	70	80	90	100
<b>6G425</b>	YKVSNRFSGV PDRFSDSGSGTDFTLRISRVEAEDLGLYFCSQSTHVPLTFGAGTKLELKR					
	#	#	#	#	#	#
<b>F(ab)-1</b>	YKVSNRFSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCSQSTHVPLTFGQGTKVEIKR					
	#	#	#	#	#	#
<b>humkI</b>	YSGSTLES GVP SRFSGSGSGTDFTLTISLQPEDFATYYCQQHNEYPLTFGQGTKVEIKR					
	==					
	+++++					
	<b>L2</b>					

	50	60	70	80	90	100	110
<b>6G425</b>	EIQLQQSGPELMKPGASVKISCKASGYFSSHYMHVWKQSHGKSLEWI						
	#	#	#	#	#	#	#
<b>F(ab)-1</b>	EVQLVESGGGLVQPGGSLRLSCAASGYFSSHYMHVWRQAPGKGLEWV						
	#						
<b>humIII</b>	EVQLVESGGGLVQPGGSLRLSCAASGFSFTGHWMNWRQAPGKGLEWV						
	=====						
	+++++						
	<b>H1</b>						

## Variable Heavy Chain Domain

	10	20	30	40
<b>6G425</b>	EIQLQQSGPELMKPGASVKISCKASGYFSSHYMHVWKQSHGKSLEWI			
	#	#	#	#
<b>F(ab)-1</b>	EVQLVESGGGLVQPGGSLRLSCAASGYFSSHYMHVWRQAPGKGLEWV			
	#			
<b>humIII</b>	EVQLVESGGGLVQPGGSLRLSCAASGFSFTGHWMNWRQAPGKGLEWV			
	=====			
	+++++			
	<b>H1</b>			

	50	a	70	80	abc	90	100	110
<b>6G425</b>	GYIDPSNGETTYNQFKGKATLTVDTSSTANVHLSSLTSDDSAVYFCAARGDYRYNGDWFFDVWGAGT							
	#	#	#	#	#	#	#	#
<b>F(ab)-1</b>	GYIDPSNGETTYNQFKGRFTISRDN SKNTLYLQMN SLRAEDTAVYYCAARGDYRYNGDWFFDVWGQGT							
	#	#	#	#	#	#	#	#
<b>humIII</b>	GMIHPSDSETRYADSVKGRFTISRDN SKNTLYLQMN SLRAEDTAVYYCAARGIYFY-GTTYFDYWGQGT							
	=====							
	+++++							
	<b>H2</b>							

	50	a	70	80	abc	90	100	110
<b>6G425</b>	GYIDPSNGETTYNQFKGKATLTVDTSSTANVHLSSLTSDDSAVYFCAARGDYRYNGDWFFDVWGAGT							
	#	#	#	#	#	#	#	#
<b>F(ab)-1</b>	GYIDPSNGETTYNQFKGRFTISRDN SKNTLYLQMN SLRAEDTAVYYCAARGDYRYNGDWFFDVWGQGT							
	#	#	#	#	#	#	#	#
<b>humIII</b>	GMIHPSDSETRYADSVKGRFTISRDN SKNTLYLQMN SLRAEDTAVYYCAARGIYFY-GTTYFDYWGQGT							
	=====							
	+++++							
	<b>H3</b>							

FIG. 29

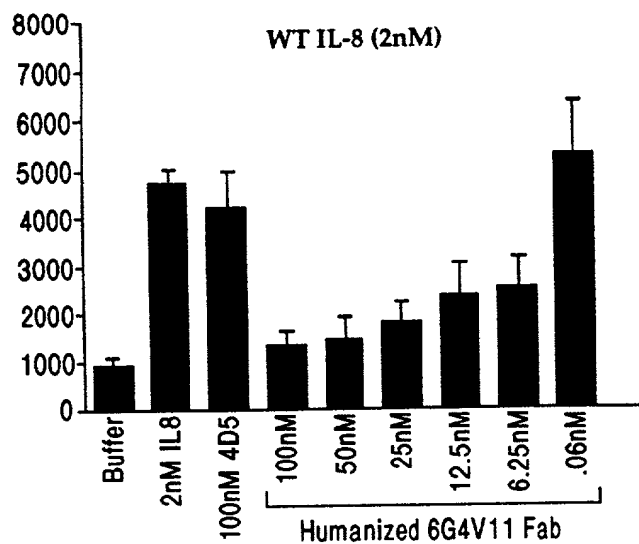


FIG. 30A

IC<sub>50</sub>~12nM

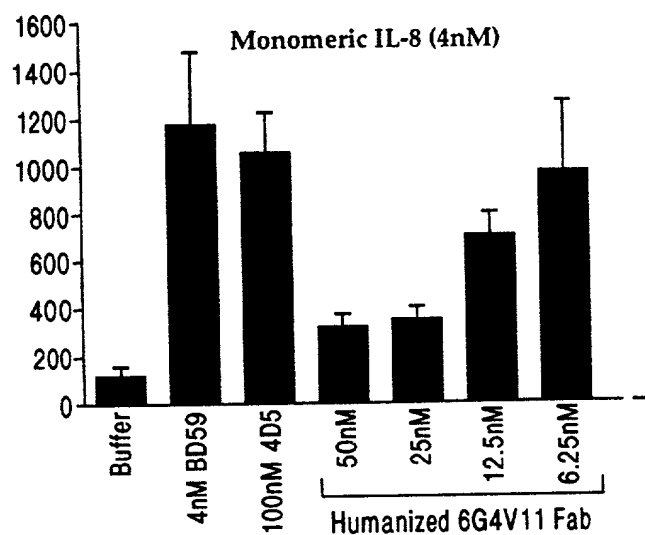


FIG. 30B

IC<sub>50</sub>~15nM

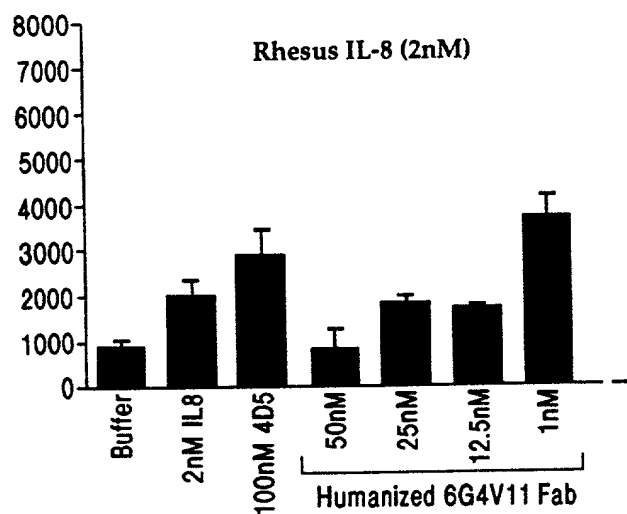


FIG. 30C

IC<sub>50</sub>~22nM

**Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V11 Light Chain**

MKKNIAFLASMFVFSIATNAYADIQMTQSPSSLSASVGDRTITCRSSQSLVHGIGNTY  
LHWYQQKPGKAPKLLIYKVSNRFSGVPSRFSGSGGTDFTLTISSLQPEDFATYYCSQST  
HVP LTFGQGTKEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN  
ALQSGNSQESVTEQDSKDSYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG  
EC

**Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V11 Heavy Chain**

MKKNIAFLASMFVFSIATNAYAEVQLVQSGGGLVQPGGSLRLSCAASGYSFSSHYMH  
WVRQAPGKGLWVGYYIDPSNGETTYNQKFKGRFTLSRDNSKNTAYLQMNSLRAEDTAVYY  
CARGDYRYNGDWFFDVWGQGTLLVTVSSASTKGP SVFPLAPSSKSTSGGTAALGCLVKDYF  
PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHNKPSNTK  
VDKKVEPKSCDKTHT

**Amino Acid Sequence of the peptide linker and M13 Phage Coat (gene-III)**

SGGSGSGDFDYKMANANKGAMTENADENALQSDAKGLDSVATDYGAIDGFIGDVS  
GLANGNGATGDFAGSSNSQMAQVGDGDN SPLMNNFRQYLP SLPQSVCECRPFVFSAGKPY  
EFSIDCDKINLFRGVFAFLLYVATFMYVFTFANILRNKES

**FIG. 31A**

1 ATGAAAAGA ATATCGCATT TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAC  
 TACTTTTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG  
 -23 M K K N I A F L L A S M F V F S I A T N  
  
 61 GCATACGCTG ATATCCAGAT GACCCAGTCC CCGAGCTCCC TGTCCGCCTC TGTGGGCGAT  
 CGTATGCGAC TATAGGTCTA CTGGGTCAGG GGCTCGAGGG ACAGGCGGAG ACACCCGCTA  
 -3 A Y A D I Q M T Q S P S S L S A S V G D  
  
 121 AGGGTCACCA TCACCTGCAG GTCAAGTCAA AGCTTAGTAC ATGGTATAGG TAACACGTAT  
 TCCAGTGGT AGTGGACGTC CAGTTCAGTT TCGAATCATG TACCATATCC ACGATGCATA  
 18 R V T I T C R S S Q S L V H G I G N T Y  
  
 181 TTACACTGGT ATCAACAGAA ACCAGGAAAA GCTCCGAAAC TACTGATTTA CAAAGTATCC  
 AATGTGACCA TAGTTGTCTT TGGTCCTTTT CGAGGCTTTG ATGACTAAAT GTTTCATAGG  
 38 L H W Y Q Q K P G K A P K L L I Y K V S  
  
 241 AATCGATTCT CTGGAGTCCC TTCTCGCTTC TCTGGATCCG GTTCTGGGAC GGATTTCACT  
 TTAGCTAAGA GACCTCAGGG AAGAGCGAAG AGACCTAGGC CAAGACCCTG CCTAAAGTGA  
 58 N R F S G V P S R F S G S G S G T D F T  
  
 301 CTGACCATCA GCAGTCTGCA GCCAGAAGAC TTCGCAACTT ATTACTGTTC ACAGAGTACT  
 GACTGGTAGT CGTCAGACGT CGGTCTTCTG AAGCGTTGAA TAATGACAAG TGTCTCATGA  
 78 L T I S S L Q P E D F A T Y Y C S Q S T  
  
 361 CATGTCCCGC TCACGTTTGG ACAGGGTACC AAGGTGGAGA TCAAACGAAC TGTGGCTGCA  
 GTACAGGGCG AGTGCAAACC TGTCCCATGG TTCCACCTCT AGTTTGCTTG ACACCGACGT  
 98 H V P L T F G Q G T K V E I K R T V A A  
  
 421 CCATCTGTCT TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCTTCTGTT  
 GGTAGACAGA AGTAGAAGGG CGGTAGACTA CTCGTCAACT TTAGACCTTG ACGAAGACAA  
 118 P S V F I F P P S D E Q L K S G T A S V  
  
 481 GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA GGTGGATAAC  
 CACACGGACG ACTTATTGAA GATAGGGTCT CTCCGGTTTC ATGTCACCTT CCACCTATTG  
 138 V C L L N N F Y P R E A K V Q W K V D N  
  
 541 GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC AGGACAGCAA GGACAGCACC  
 CGGGAGGTTA GCCCATAGAG GGTCTCTCTA CAGTGTCTCG TCCTGTCTGT CCTGTCTGTG  
 158 A L Q S G N S Q E S V T E Q D S K D S T  
  
 601 TACAGCCTCA GCAGCACCCT GACGCTGAGC AAAGCAGACT ACGAGAAACA CAAAGTCTAC  
 ATGTCGGAGT CGTCGTGGGA CTGCGACTCG TTTCTGTCTGA TGCTCTTTGT GTTTCAGATG  
 178 Y S L S S T L T L S K A D Y E K H K V Y  
  
 661 GCCTGCGAAG TCACCCATCA GGGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA  
 CGGACGCTTC AGTGGGTAGT CCCGGAAGTC AGCGGGCAGT GTTCTCTGAA GTTGTCCCTT  
 198 A C E V T H Q G L S S P V T K S F N R G  
  
 721 GAGTGTAAAG CTGATCCTCT ACGCCGGACG CATCGTGGCC CTAGTACGCA ACTAGTCGTA  
 CTCACAATTC GACTAGGAGA TGCGGCCTGC GTAGCACCAG GATCATGCGT TGATCAGCAT  
 218 E C O

FIG. 31B

**Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V19 Light Chain**

MKKNIAFLLASMFVFSIATNAYADIQMTQSPSSLSASVGDRTITCRSSQSLVHGIGNTY  
LHWYQQKPGKAPKLLIYKVSNRFSGVPSRFSGSGTDFTLTISSLQPEDFATYYCSQST  
HVPLTFTGGQGTKVEIKRTVAAPSVEIFPPSDEQLKSGTASVVCCLNNFYPREAKVQWKVDN  
ALQSGNSQESVTEQDSKDSITYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG  
EC

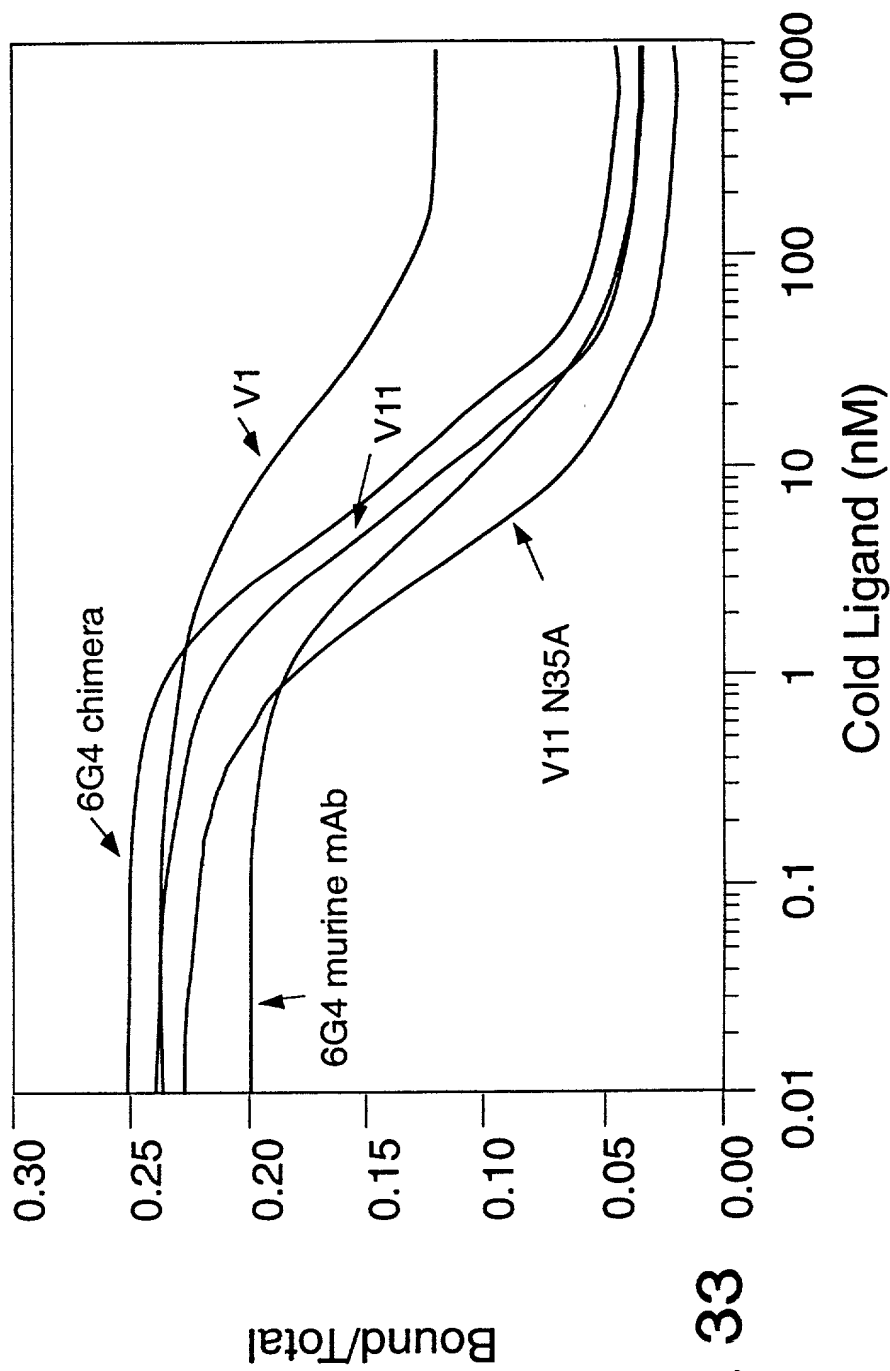
**Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V19 Heavy Chain**

MKKNIAFLLASMFVFSIATNAYAEVQLVESGGGLVQPGGSLRLSCAASGYSFSSHYMH  
WVKQAPGKGLEWVGYYIDPSNGETTYNQKFKGRFTLSRDNSKNTAYLQMNSLRAEDTAVYY  
CARGDYRYNGDWFFDVGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF  
PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSSLGTQTYICNVNHHKPSNTK  
VDKKVEPKSCDKTHT

**FIG. 31C**







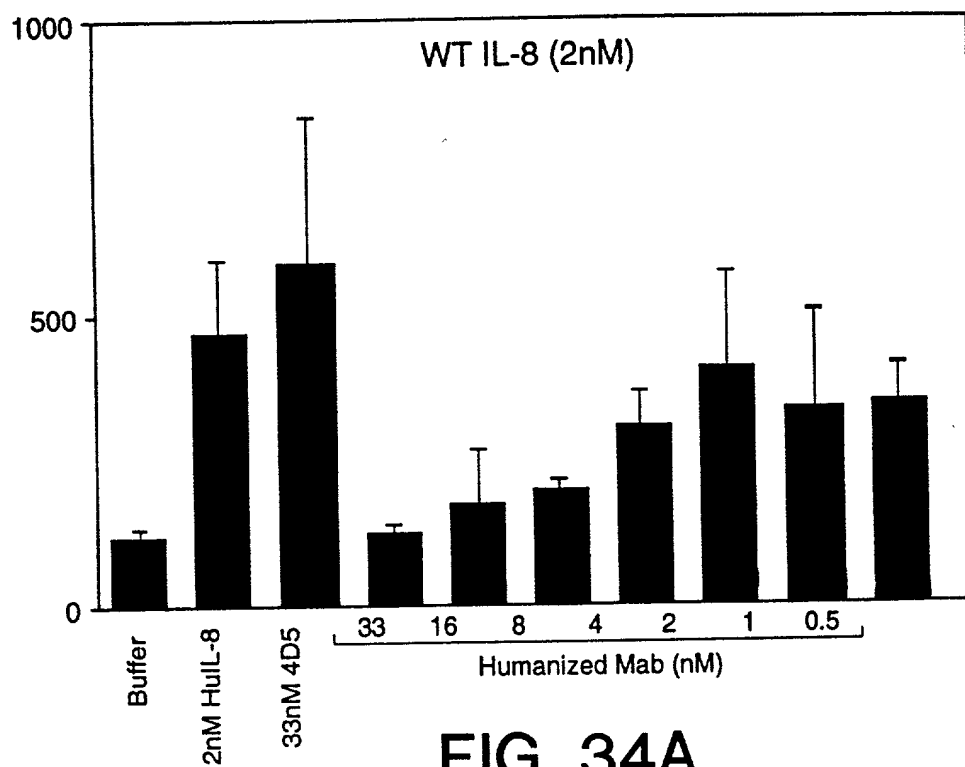


FIG. 34A

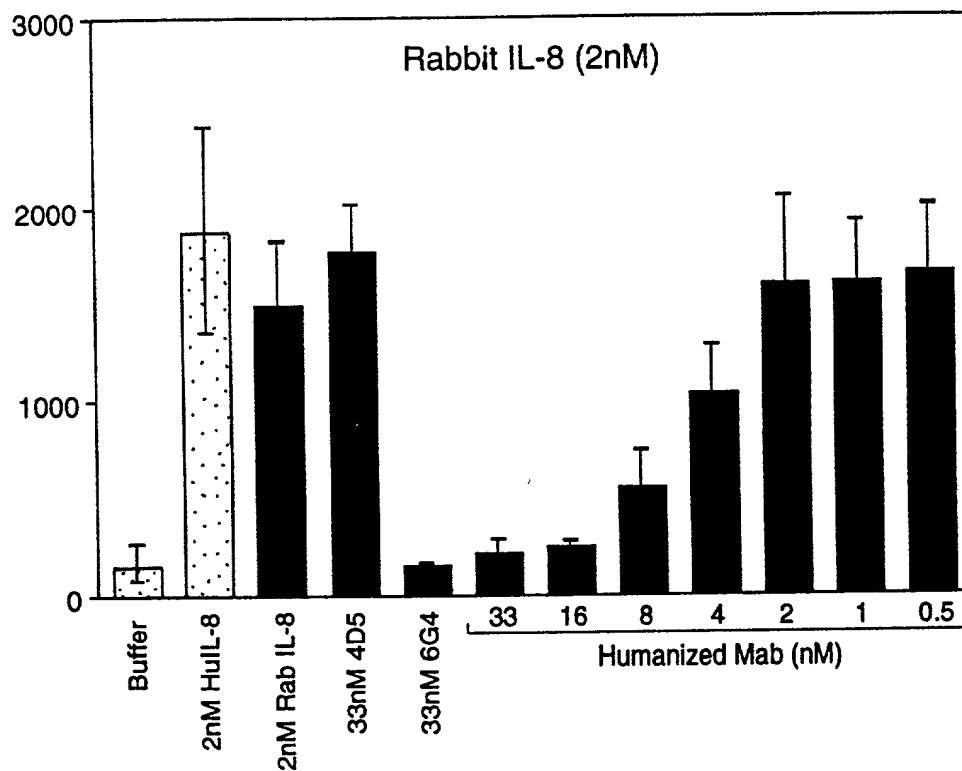
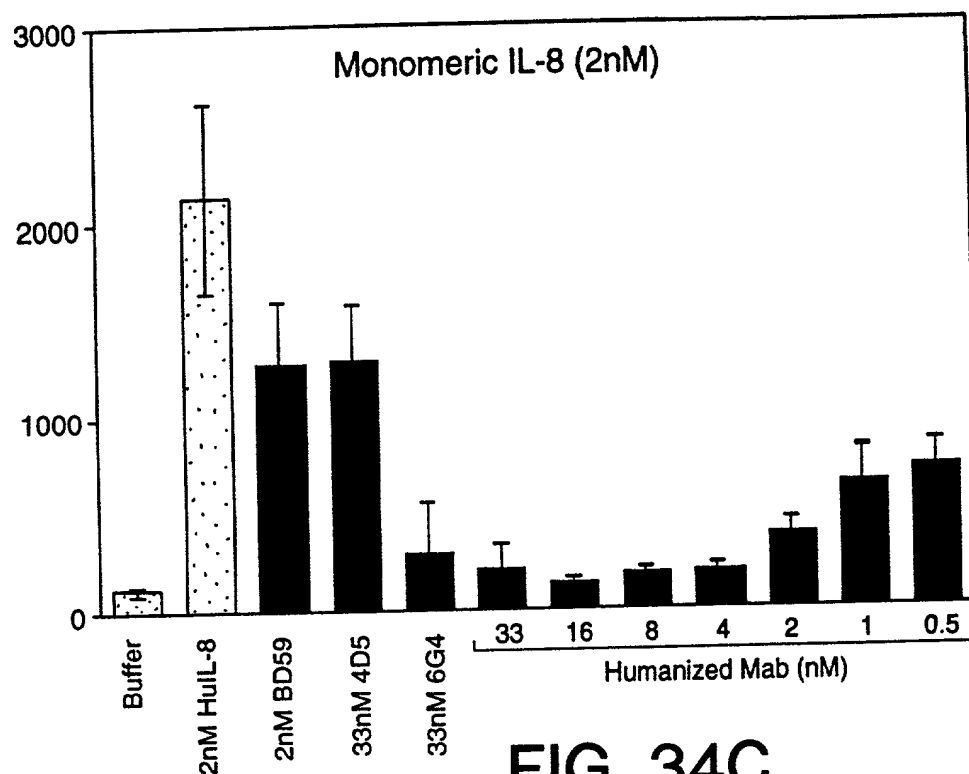
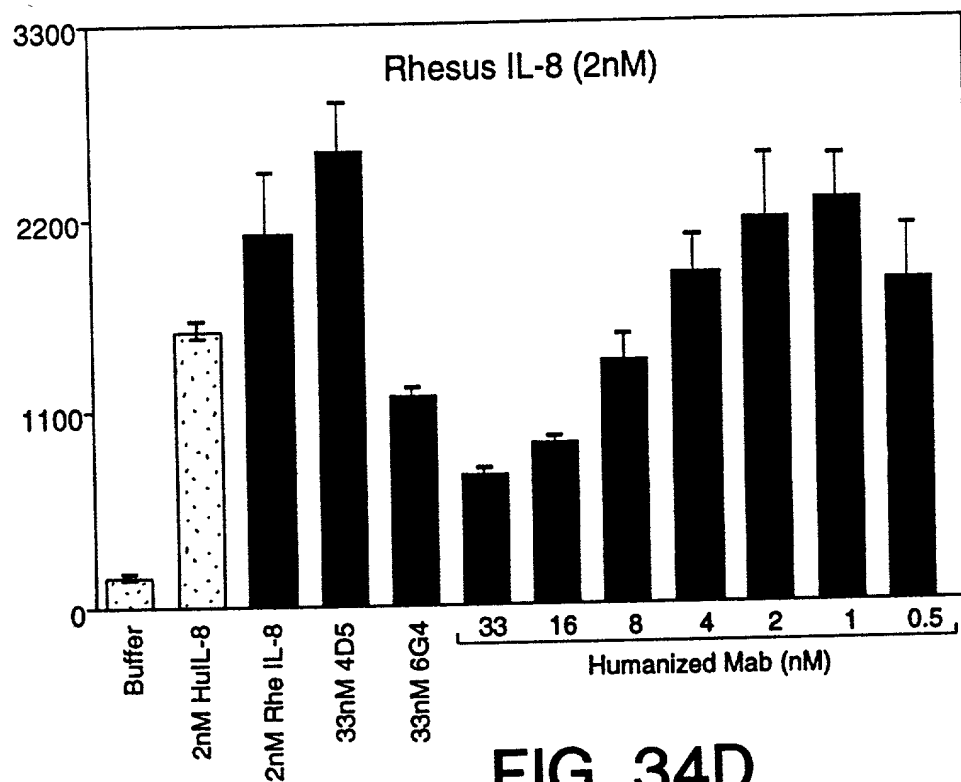


FIG. 34B



**FIG. 34C**



**FIG. 34D**

# **Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V11N35A Light Chain**

MKKNIAFLASMFVFSIATNAYADIQMTQSPSSLSASVGDRTITCRSSQSLVHGIGATY  
LHWYQQKPGKAPKLLIYKVSNRFSGVPSRFSGSGTDFTLTISSLPEDFATYYCSQST  
HVPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVCLLNFFYPREAKVQWKVDN  
ALQSGNSQESVTEQDSKDSYSTLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG  
EC

# **Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V11N35A Heavy Chain**

MKKNIAFLASMFVFSIATNAYAEVQLVQSGGGLVQPGGSLRLSCAASGYSFSSHYMH  
WVRQAPGKGLEWVGYIDPSNGETTYNQKFKGRFTLSRDNSKNTAYLQMNSLRAEDTAVYY  
CARGDYRYNGDWFFDVWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF  
PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK  
VDKKVEPKSCDKTHT

# **Amino Acid Sequence of the putative Pepsin Cleavage Site and GCN4 Leucine Zipper**

CPPCPAPELLLGGRMKQLEDKVEELL SKNYHLENEVARLKKLVGER

**FIG. 35**

1 ATGAAAAAGA ATATCGCATT TCTTCTTGCA TCTATGTTTCG TTTTCTCTAT TGCTACAAAC  
 TACTTTTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG  
 -23 M K K N I A F L L A S M F V F S I A T N  
 61 GCATACGCTG ATATCCAGAT GACCCAGTCC CCGAGCTCCC TGTCGCGCTC TGTGGGCGAT  
 CGTATGCGAC TATAGGTCTA CTGGGTCAGG GGCTCGAGGG ACAGGCGGAG ACACCCGCTA  
 -3 A Y A D I Q M T Q S P S S L S A S V G D  
 121 AGGGTCACCA TCACCTGCAG GTCAAGTCAA AGCTTAGTAC ATGGTATAGG TGCTACGTAT  
 TCCCAGTGGT AGTGGACGTC CAGTTCAGTT TCGAATCATG TACCATATCC ACGATGCATA  
 18 R V T I T C R S S O S L V H G I G A T Y  
 181 TTACACTGGT ATCAACAGAA ACCAGGAAAA GCTCCGAAAC TACTGATTTA CAAAGTATCC  
 AATGTGACCA TAGTTGTCTT TGGTCCTTTT CGAGGCTTTG ATGACTAAAT GTTTCATAGG  
 38 L H W Y Q Q K P G K A P K L L I Y K V S  
 241 AATCGATTCT CTGGAGTCCC TTCTCGCTTC TCTGGATCCG GTTCTGGGAC GGATTTCACT  
 TTAGCTAAGA GACCTCAGGG AAGAGCGAAG AGACCTAGGC CAAGACCTCG CCTAAAGTGA  
 58 N R F S G V P S R F S G S G S G T D F T  
 301 CTGACCATCA GCAGTCTGCA GCCAGAAGAC TTCGCAACTT ATTACTGTTC ACAGAGTACT  
 GACTGGTAGT CGTCAGACGT CGGTCTTCTG AAGCGTTGAA TAATGACAAG TGTCTCATGA  
 78 L T I S S L Q P E D F A T Y Y C S O S T  
 361 CATGTCCCGC TCACGTTTGG ACAGGGTACC AAGGTGGAGA TCAAACGAAC TGTGGCTGCA  
 GTACAGGGCG AGTGCAAACC TGTCCCATGG TTCCACCTCT AGTTTGCTTG ACACCGACGT  
 98 H V P L T F G Q G T K V E I K R T V A A  
 421 CCATCTGTCT TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCTTCTGTT  
 GGTAGACAGA AGTAGAAGGG CGGTAGACTA CTCGTCAACT TTAGACCTTG ACGAAGACAA  
 118 P S V F I F P P S D E Q L K S G T A S V  
 481 GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA GGTGGATAAC  
 CACACGGACG ACTTATTGAA GATAGGGTCT CTCCGGTTTC ATGTCACCTT CCACCTATTG  
 138 V C L L N N F Y P R E A K V Q W K V D N  
 541 GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC AGGACAGCAA GGACAGCACC  
 CGGGAGGTTA GCCCATTGAG GGTCCTCTCA CAGTGTCTCG TCCTGTGCTT CCTGTCTGTT  
 158 A L Q S G N S Q E S V T E Q D S K D S T  
 601 TACAGCCTCA GCAGCACCTT GACGCTGAGC AAAGCAGACT ACGAGAAACA CAAAGTCTAC  
 ATGTCGGAGT CGTCGTGGGA CTGCGACTCG TTTCGTCTGA TGCTCTTTGT GTTTCAGATG  
 178 Y S L S S T L T L S K A D Y E K H K V Y  
 661 GCCTGCGAAG TCACCCATCA GGGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA  
 CGGACGCTTC AGTGGGTAGT CCCGGAAGTC AGCGGGCAGT GTTCTCTGAA GTTGTCCCCT  
 198 A C E V T H Q G L S S P V T K S F N R G  
 721 GAGTGTTAAG CTGATCCTCT ACGCCGGACG CATCGTGGCC CTAGTACGCA ACTAGTCGTA  
 CTCACAATTC GACTAGGAGA TGCGGCCTGC GTAGCACCGG GATCATGCGT TGATCAGCAT  
 218 E C O

FIG. 36

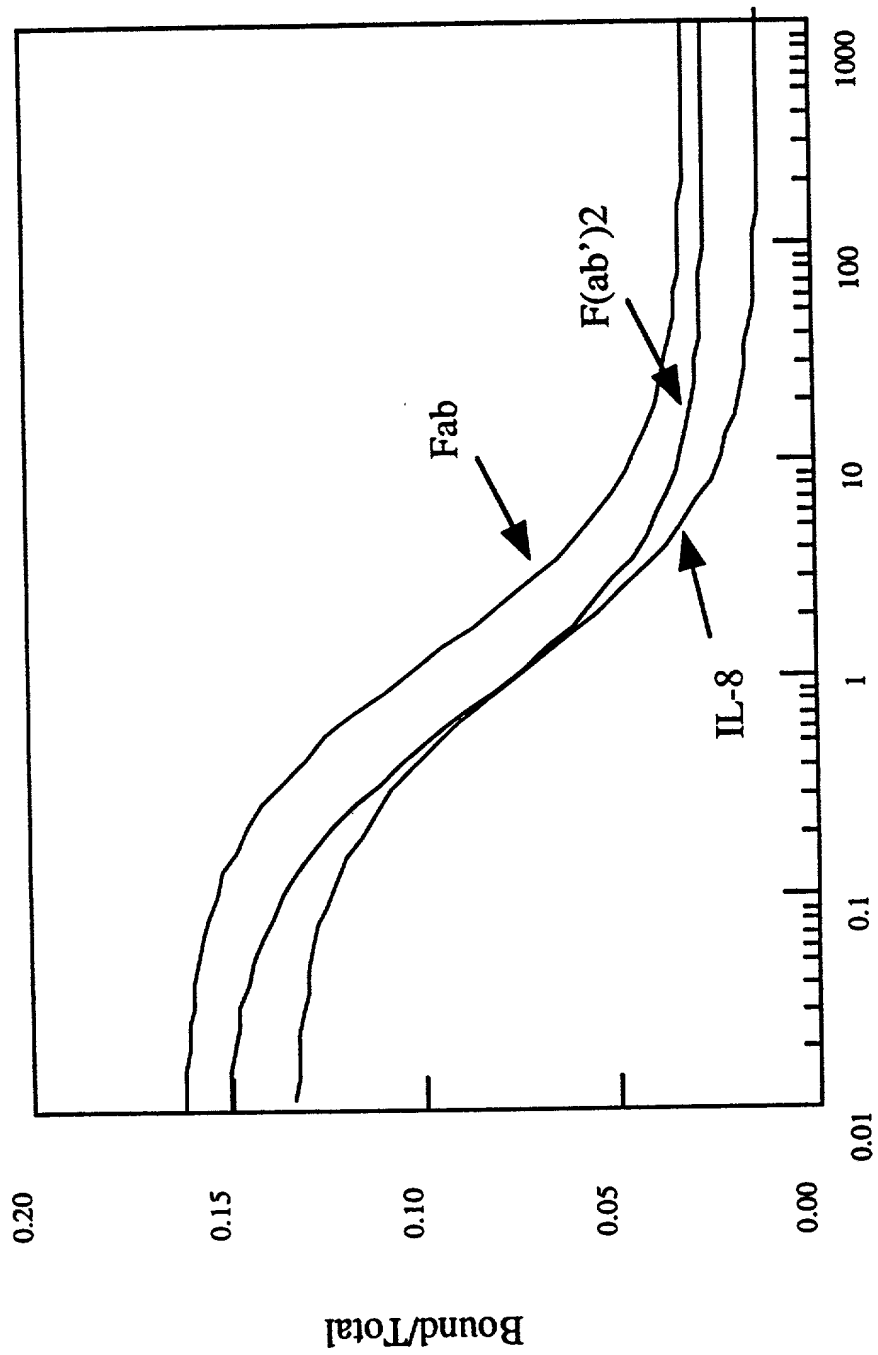
781 AAAAGGGTAT CTAGAGGTTG AGGTGATTTT ATGAAAAAGA ATATCGCATT TCTTCTTGCA  
 TTTTCCCATG GATCTCCAAC TCCACTAAAA TACTTTTTTCT TATAGCGTAA AGAAGAACGT  
 -1 M K K N I A F L L A  
 841 TCTATGTTTCG TTTTCTCTAT TGCTACAAAC GCGTACGCTG AGGTTTCAGCT AGTGCAGTCT  
 AGATACAAGC AAAAAAGATA ACGATGTTTG CGCATGCGAC TCCAAGTCGA TCACGTCAGA  
 -11 S M F V F S I A T N A Y A E V Q L V Q S  
 901 GGCGGTGGCC TGGTGCAGCC AGGGGGCTCA CTCCGTTTGT CCTGTGCAGC TTCTGGCTAC  
 CCGCCACCGG ACCACGTCGG TCCCCGAGT GAGGCAAACA GGACACGTCG AAGACCGATG  
 8 G G G L V Q P G G S L R L S C A A S G Y  
 961 TCCTTCTCGA GTCACTATAT GCACTGGGTC CGTCAGGCCC CGGGTAAGGG CCTGGAATGG  
 AGGAAGAGCT CAGTGATATA CGTGACCCAG GCAGTCCGGG GCCCATTCCC GGACCTTACC  
 28 S F S S H Y M H W V R Q A P G K G L E W  
 1021 GTTGGATATA TTGATCCTTC CAATGGTGAA ACTACGTATA ATCAAAAGTT CAAGGGCCGT  
 CAACCTATAT AACTAGGAAG GTTACCACTT TGATGCATAT TAGTTTTCAA GTTCCCGGCA  
 48 V G Y I D P S N G E T T Y N O K F K G R  
 1081 TTCACCTTAT CTCGCGACAA CTCCAAAAAC ACAGCATACC TGCAGATGAA CAGCCTGCGT  
 AAGTGAAATA GAGCGCTGTT GAGGTTTTTG TGTCGTATGG ACGTCTACTT GTCGGACGCA  
 68 F T L S R D N S K N T A Y L Q M N S L R  
 1141 GCTGAGGACA CTGCCGTCTA TTACTGTGCA AGAGGGGATT ATCGCTACAA TGGTGACTGG  
 CGACTCCTGT GACGGCAGAT AATGACACGT TCTCCCTTAA TAGCGATGTT ACCACTGACC  
 88 A E D T A V Y Y C A R G D Y R Y N G D W  
 1201 TTCTTCGACG TCTGGGGTCA AGGAACCCTG GTCACCGTCT CCTCGGCCCTC CACCAAGGGC  
 AAGAAGCTGC AGACCCAGT TCCTTGGGAC CAGTGGCAGA GGAGCCGGAG GTGGTTCCCG  
 108 F F D V W G Q G T L V T V S S A S T K G  
 1261 CCATCGGTCT TCCCCCTGGC ACCCTCCTCC AAGAGCACCT CTGGGGGCAC AGCGGCCCTG  
 GGTAGCCAGA AGGGGGACCG TGGGAGGAGG TTCTCGTGGA GACCCCGTG TCGCCGGGAC  
 128 P S V F P L A P S S K S T S G G T A A L  
 1321 GGCTGCCTGG TCAAGGACTA CTTCCCCGAA CCGGTGACGG TGTCGTGGAA CTCAGGCGCC  
 CCGACGGACC AGTTCCTGAT GAAGGGGCTT GGCCACTGCC ACAGCACCTT GAGTCCGCGG  
 148 G C L V K D Y F P E P V T V S W N S G A  
 1381 CTGACCAGCG GCGTGCACAC CTTCCCGGCT GTCCTACAGT CCTCAGGACT CTACTCCCTC  
 GACTGGTTCG CGCACGTGTG GAAGGGCCGA CAGGATGTCA GGAGTCCTGA GATGAGGGAG  
 168 L T S G V H T F P A V L Q S S G L Y S L  
 1441 AGCAGCGTGG TGACCGTGCC CTCCAGCAGC TTGGGCACCC AGACCTACAT CTGCAACGTG  
 TCGTCGCACC ACTGGCACGG GAGGTCGTG AACCCTGGG TCTGGATGTA GACGTTGCAC  
 188 S S V V T V P S S S L G T Q T Y I C N V  
 1501 AATCACAAGC CCAGCAACAC CAAGGTCGAC AAGAAAGTTG AGCCCAAATC TTGTGACAAA  
 TTAGTGTTTC GGTGCTGTG GTTCCAGCTG TTCTTTCAAC TCGGGTTTGA AACACTGTTT  
 208 N H K P S N T K V D K K V E P K S C D K  
 1561 ACTCACACAT GCGCGCCGTG CCCAGCACCA GAACTGCTGG GCGGCCGCAT GAAACAGCTA  
 TGAGTGTGTA CGGGCGGCAC GGGTCGTGGT CTTGACGACC CGCCGGCGTA CTTTGTGCGT  
 228 T H T C P P C P A P E L L G G R M K Q L

FIG. 37A

1621 GAGGACAAGG TCGAAGAGCT ACTCTCCAAG AACTACCACC TAGAGAATGA AGTGGCAAGA  
CTCCTGTTCC AGCTTCTCGA TGAGAGGTTC TTGATGGTGG ATCTCTTACT TCACCGTTCT  
248 E D K V E E L L S K N Y H L E N E V A R

1681 CTCAAAAAGC TTGTCGGGGA GCGCTAA  
GAGTTTTTTCG AACAGCCCCT CGCGATT  
268 L K K L V G E R O

FIG. 37B



Cold Ligand (nM)

FIG. 38



FIG. 39

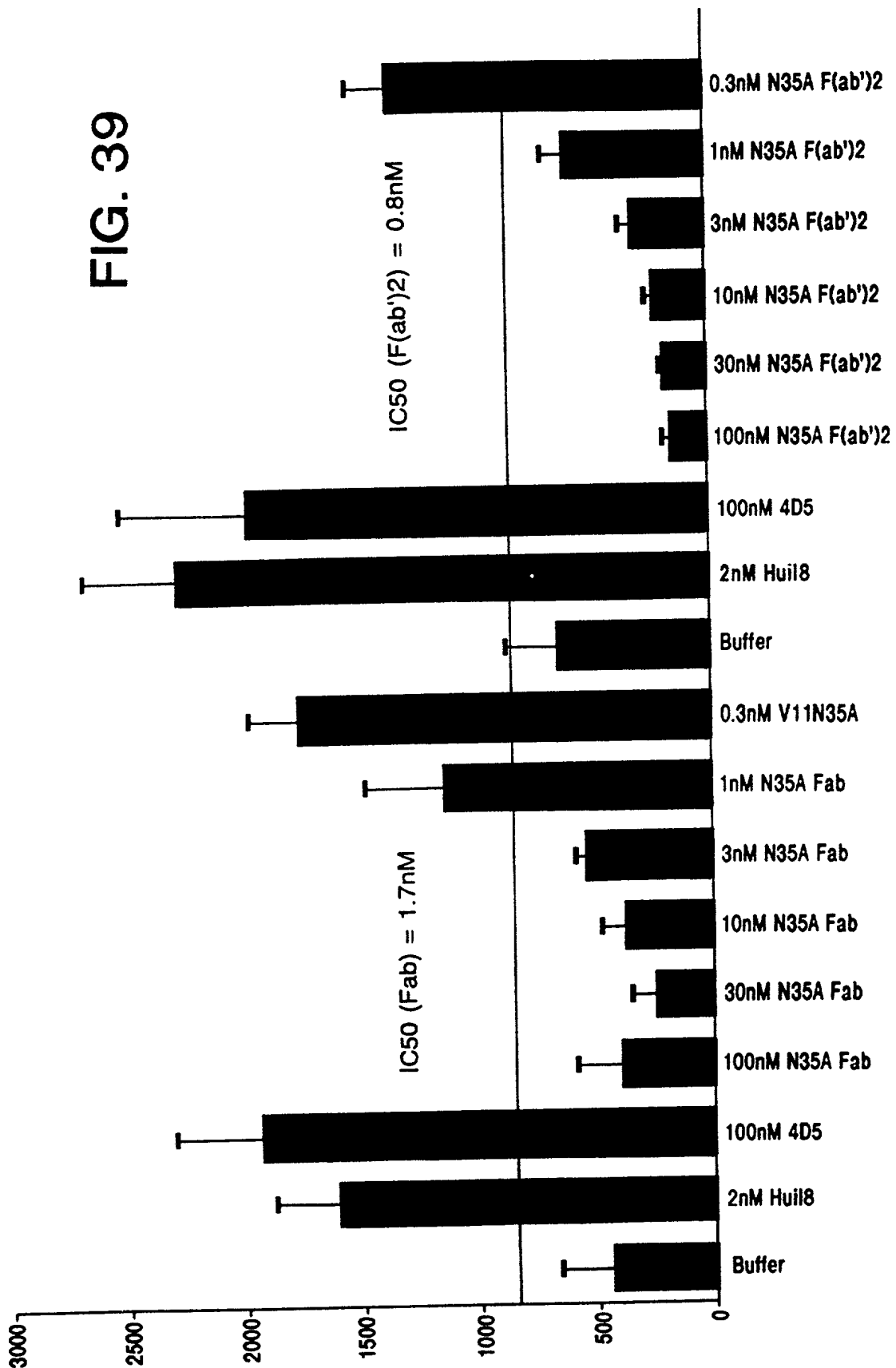
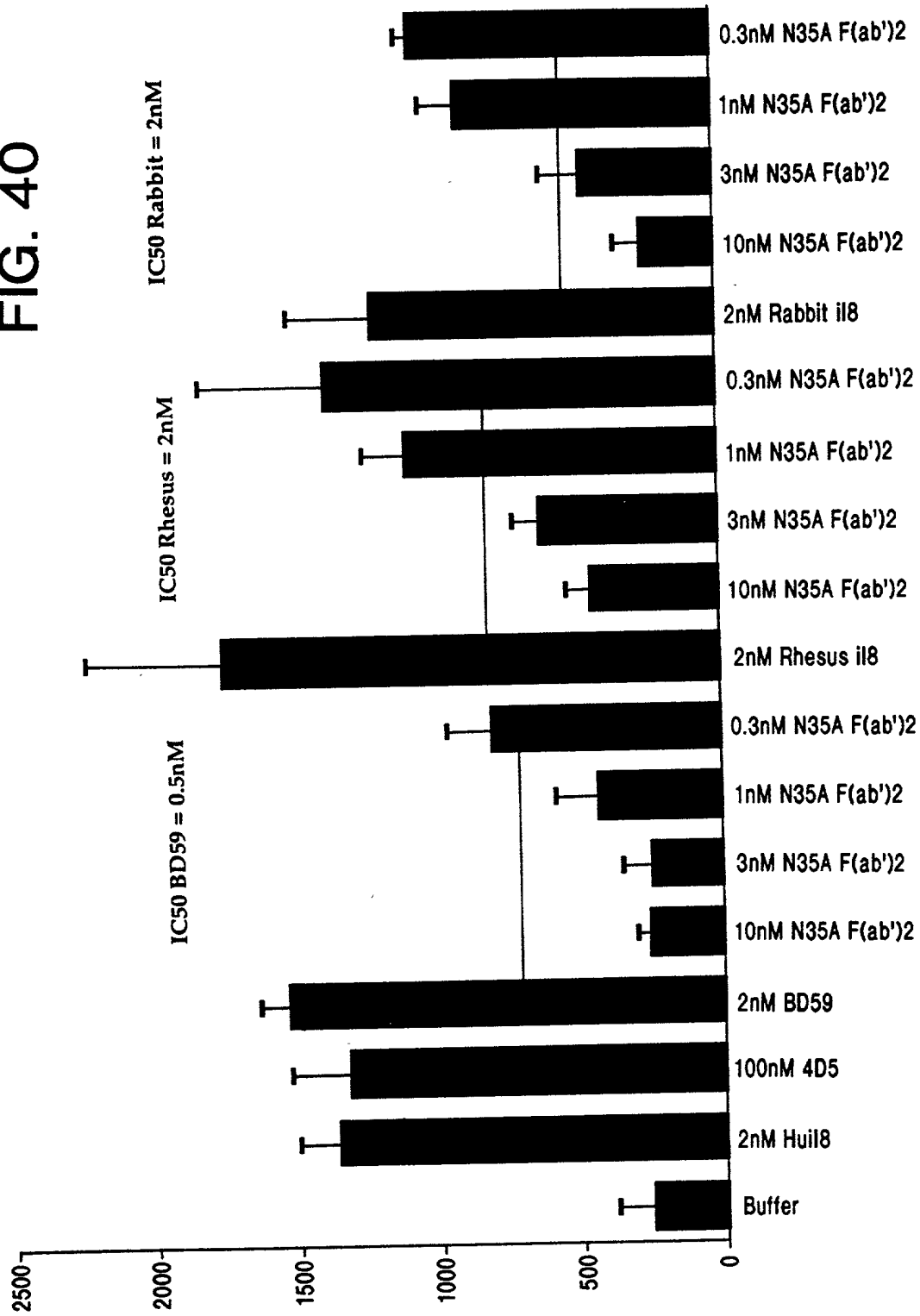


FIG. 40



ecori pflMI plei  
 apoI bslI mboII taqI  
 1 GAATCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAATATC TCATTGCTGA GTTGTATTTT AAGCTTGCCC AAAAAGAGA AGAGTCGAAT  
 CTTAAGTTGA AGAGGTATGA AACCTATTCC TTTATGCTGT TACTTTTTAG AGTAACGACT CAACAATAAA TTGGAACGGG TTTTCTCTCT TCTCAGCTTA

bspMI  
 hinPI mboI/ndeII[dam-]  
 hhaI/cfoI dpnI[dam+]  
 mstI aluI dpnII[dam-]  
 aviII/fspI hindIII bclI[dam-] mnlI  
 101 GAAGTGTG CGCAGGTAGA ACCTTTGGAG ATTATCGTCA CTGCAATGCT TCGCAATATG GCGCAAAATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG  
 CTTGACACAC GCGTCCATCT TCGAAACCTC TAATAGCAGT GAGGTTACGA AGCGTTATAC GCGGTTTTAC TGGTTGTCGC CAACTAACA GTCCATCTCC

rsal  
 hinPI  
 hhaI/cfoI mnlI  
 haeII csp6I cac8I  
 201 GGGCGGTGTA CGAGGTAAAG CCCGATGCCA GCATTCCCTGA CGACGATACG GAGGTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTGAGTA  
 CCCGCGACAT GCTCCATTTC GGGCTACGGT CGTAAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GACGAGTCAT

truuI  
 maeIII  
 apoI  
 301 AAAGTTAAT CTTTTCACA GCTGTCAATA AGTTGTCACG GCCGAGACTT ATAGTCGCTT TGTGTTTATT TTTTAATGTA TTTGTAAC TAATTCGAGC  
 TTTTCAATTA GAAAAGTTGT CGACAGTATT TCAACAGTGC CGGCTCTGAA TATCAGCGAA ACAAATAA AAATATCAT AACATTCAT CTTAAGCTCG

alul  
 hindIII  
 tru9I mseI cac8I  
 ddeI bsrDI  
 nlaIII  
 mslI  
 maeIII bsrDI  
 hinPI  
 hhaI/cfoI  
 bbvI  
 fnu4HI bstUI snaBI  
 bsoFI bsh1236I  
 bbvI hinPI bsaAI  
 aluI hhaI/cfoI  
 maeII  
 fnuDII/mvni  
 fnu4HI  
 bsoFI  
 bbvI  
 maeII  
 mnlI  
 foki  
 sfaNI  
 aluI  
 sstI  
 sacI  
 hgiJII  
 hgiAI/aspHI  
 ecll36II  
 ecorI bsp1286  
 rmaI bsiHKAI  
 maeI bmyI  
 bfaI taqI  
 tru9I  
 mseI  
 maeIII apoI banII  
 TTTGTAAC TAATTCGAGC  
 AACATTCAT CTTAAGCTCG

FIG. 41A







rmaI  
 maeI  
 bfaI  
 xbaI  
 mnlI  
 hpaI  
 mnlI  
 rsaI  
 csp6I  
 speI  
 1201 AGTACGCAAC TAGTCGTAAA AAGGGTATCT AGAGGTTGAG GTGATTTTAT GAAAAAGAAAT ATCGCATTTT TTCTGTCATC TATGTTTCGT TTTTCTATTG  
 TCATGCGTTG ATCAGCATTT TTCCCATAGA TCTCCAAC TC CACTAAAAATA CTTTTTCTTA TAGCGTAAAG AAGAAGCTAG ATACAAGCAA AAAAGATAAC  
 M K K N I A F L L A S M F V F S I A  
 scrFI  
 mvaI  
 ecorII  
 dsav  
 scrFI  
 mvaI  
 fnu4HI  
 ecorII  
 dsav  
 bstNI  
 bsoFI  
 bsp1286  
 apyI[dcm+] bsaJI bmyI  
 haeIII/palI apyI[dcm+]  
 aciI haeI  
 bbvI  
 banII  
 aluI  
 alwNI[dcm-]  
 fnu4HI  
 bsoFI  
 bbvI  
 1301 CTACAAACGC GTACGCTGAG GTTCAGCTGAG TGCAGTCTGG CGGTGGGCTG GTGCAGCCAG GGGGCTCACT CCGTTTGTC TGTGCAGCTT CTGGCTACTC  
 GATGTTTGGC CATGGGACTC CAAGTCGATC ACGTCAAGCC GCCACCGGAC CAGTCGCTC CCCCAGTGA GGCAACAGG ACACGTCGAA GACCGATGAG  
 -5 T N A Y A E V Q L V Q S G G L V Q P G G S L R L S C A A S G Y S  
 scrFI  
 nciI  
 mspI  
 hpaII  
 dsav  
 caulI  
 bsII  
 xmaI/pspAI  
 smaI  
 scrFI  
 nciI  
 dsav  
 caulI  
 bsII  
 scrFI  
 mvaI  
 ecorII

FIG. 41E

1401 CTTCTCGAGT CACTATATGC ACTGGGTCGG TCAGGCCCGG GGTAAGGGCC TGGAAATGGGT TGGATATATT GATCCTTCCA ATGGTGAAC TACGTATAAT  
GAAGAGCTCA GTGATATACG TGACCCAGGC AGTCGGGGC CCATTCCCGG ACCTTACCCA ACCTATATAA CATAGGAAGT TACCACCTTG ATGCATATTA  
29 F S S H Y M H W V R Q A P G K G L E W V G Y I D P S N G E T T Y N

1501 CAAAAGTTCA AGGGCGGTTT CACTTTATCT GCGGACAAC CCAAAAACAC AGCATACCTG CAGATGAACA GCCTGCGTCG TGAGGACACT GCCGTCTATT  
GTTTCAAGT TCCCGGCAAA GTGAATAGA GCGCTGTTGA GGTTTTGTG TGTATGGAC GTCTACTGT CCGACGACAG ACTCCTGTGA CGGCAGATAA  
62 Q K F K G R F T L S R D N S K N T A Y L Q M N S L R A E D T A V Y Y

1601 ACTGTGCAAG AGGGGATTAT CGCTACAATG GTGACTGGTT CTTCCAGCGTC TGGGCTCAAG GAACCTCTGGT CACCGTCTCC TCGGCCCTCCA CCAAGGGCCC  
TGACACGTTT TCCCTTAATA GCGATGTTAC CACTGACCAA GAAGTGCAG ACCCAGTTC CTTGGGACCA CTTGGCAGAGG AGCCGGAGGT GGTTCGCCGG  
96 C A R G D Y R Y N G D W F F D V W G Q G T L V T V S S A S T K G P

seq right is from p6G425chim2.fab2

**FIG. 41F**

















[illegible]

**FIG. 41N**



[illegible]

FIG. 410

hgiAI/aspHI  
 bsp1286  
 bsiHKA1  
 bmyI ndeI  
 apaLI/snoI  
 alw44I/snoI  
 ddeI  
 rsaI  
 csp6I  
 AGAGTGCACC  
 CATCAGAGCA GATTGTACTG  
 CTAAACATGAC TCTCACGTGG  
 4401 CGGGTGTCTGG  
 GCGCCAGCC CCGGTCGGT ACTGGGTCTAG TGCATCGCTA TCGCCTCACA TATGACCGAA TTGATACGCC GTAGTCTCGT  
 4501 ATATCGCGTG TGAATAACCG CACAGATGCG TAAGGAGAAA ATACCGCATC AGCGCTCTT CCGCTTCTC GCTCACTGAC TCGTGGCT CGTCTGTTCCG  
 TATACGCCAC ACTTTATGGC GTGTCTACGC ATTCTCTTT TATGGCGTAG TCCGCGAGAA GCGAAGGAG CGAGTGACTG AGCGACGCGA GCCAGCAAGC  
 4601 GCTGCGGCGA GCGGTATCAG CTCACCTCAA GCGCGTAATA CGGTTATCCA CAGAAATCAGG GGATAACGCA GGAAAGAAC TGTGACCAA AGGCCAGCAA  
 TACGCGCGCT GCCATAGTC GAGTGAGTTT CCGCCATTAT GCCAATAGGT GTCTTAGTCC CCTATGCGT CCTTTCTTGT ACACCTCGTT TCCGTCGTT  
 4701 AAGGCCAGGA ACCGTAAAAA GCGCGCGTTG CTGGCGTTT TCCATAGGCT CCGCCCCCTT GACGAGCATC ACAAATCTG ACCTCAAGT CAGAGTGGC  
 TTCGCGTCTT TGGCATTTT CCGGCGCAAC GACCCGCAAA AGGTATCCGA GCGGGGGGA CTGCTCGTAG TGTTTTTCG TCGGAGTTCA GTCTCCACCG

FIG. 41P



5401 TCTGCTGAAG CCAGTTACCT TCGGAARAAG AGTTGGTAGC TCTTGATCCG GCAACAAAC CACCGCTGGT ACCTGCTGTT TTTTGTGTTG CAAGCAGCAG  
AGACGACTTC GGTCATGGA AGCCTTTTTC TCAACCATCG AGAACTAGGC CGTTTGTGTTG GTGGCGACCA TCGCCACCAA AAAACAAC GTTCTGTCGTC

maeIII  
eco57I bsrI  
mspI  
hpaII  
sau3AI  
mboI/ndeII[dam-]  
dpmI[dam+]  
dpmII[dam-]  
alwI[dam-]  
nspBII  
aciI  
aciI  
cac8I  
fnu4HI  
bscFI  
bbvI  
5201 TCTGCTGAAG CCAGTTACCT TCGGAARAAG AGTTGGTAGC TCTTGATCCG GCAACAAAC CACCGCTGGT ACCTGCTGTT TTTTGTGTTG CAAGCAGCAG  
AGACGACTTC GGTCATGGA AGCCTTTTTC TCAACCATCG AGAACTAGGC CGTTTGTGTTG GTGGCGACCA TCGCCACCAA AAAACAAC GTTCTGTCGTC

sau3AI  
sau3AI mboI/ndeII[dam-]  
mboI/ndeII[dam-]  
sau3AI mboII[dam-] dpmI[dam+]  
mboI/ndeII[dam-] dpmII[dam-]  
dpmI[dam+]  
dpmII[dam-]  
bstYI/xhoII alwI[dam-]  
alwI[dam-] bstYI/xhoII  
alwI[dam-] bstYI/xhoII  
hgaI ddeI  
tru9I  
mseI  
maeII  
nlaIII  
rcal  
bspHI  
5301 ATTACGCGCA GAAAGGATC ATCTCAAGAA GATCCTTGA TCTTTTCTAC GGGGTCTGAC GCTCAGTGA ACAGAACTC ACCTTAAGG ATTTTGGTCA  
TAATGCGCGT CTTTITTTCC TAGAGTTCTT CTAGGAAACT AGAAAGATG CCCCAGACTG CGAGTCACCT TGCTTTTGAG TGAATTTCCC TAAACCCAGT

sau3AI  
mboI/ndeII[dam-]  
rmaI  
hphI dpmI[dam+]  
mboII[dam-]  
sau3AI maeI tru9I  
mboI/ndeII[dam-] mseI  
dpmI[dam+]  
dpmII[dam-] alwI[dam-] tru9I  
bstYI/xhoII bstYI/xhoII mseI  
alwI[dam-] bfaI ahaII/draI  
5401 TGAGATTATC AAAAGGATC TTCACCTAGA TCCTTTTAAA TTAAATATGA AGTTTAAAT CAATCTAAAG TATATATGAG TAAACTTGGT CTGACAGTGA  
ACTCTAATAG TTTTTCCTAG AAGTGGATCT AGGAAATTT AATTTTACT TCNAAATTTA GTTAGATTTC ATATATACTC ATTTGAACCA GACTGTCAAT

nlaIV  
hgiCI  
banI  
mnlI  
tru9I  
mseI  
pleI  
hinFI  
ahdI/eam1105I  
fokI  
5501 CCAATGCTTA ATCAGTGAGG CACCTATCTC AGCGATCTGT CTATTTCTGT CATCCATAGT TGCCTGACTC CCCGTCTGT AGATAACTAC GATACGGGAG  
GGTTACGAAT TAGTCACTCC GTGGATAGAG TCGCTAGACA GATAAAGCAA GTAGGTATCA ACGGACTGAG GGCAGACCA TCTATTGATG CTATGCCCTC

FIG. 41R

5601 GGCTTACCAT CTGGCCCCAG TGCTGCAATG ATACCGCGAG ACCCAGGCTC ACCGGCTCCA GATTATATCAG CAATAAACCA GCCAGCCGGA AGGGCCGAGC  
CCGNAATGGTA GACCGGGGTC ACACGCTTAC TATGGCGGTC TGGGTCGAG TGGCGGAGT CTAAATAGTC GTTATTGCT CCGTCCGCTC TCCCGGCTCG

5701 GCAGAAATGG TCCTGCAACT TTATCCGCCT CCATCCAGTC TATTAATTCT TGCCGGGAAG CTAGAGTAAG TAGTTCGCCA GTTAATAGTT TCGGCAACGT  
CGTCTTACC AGGACGTGA AATAGGCGGA GGTAGGTCAG ATAATTAACA ACGGCCCTTC GATCTCATTC ATCAAGCGGT CAATTATCAA ACGGTTGCA

5801 TGTTGCCATT GCTGCAGGCA TCGTGGTGTC ACGCTCCGTC TTTGGTATGG CTTTCATTCAG CTCGGGTTCC CAACGATCAA GCGGAGTTAC ATGATCCCCC  
ACAACGGTAA CGACGTCCTT AGCACCACAG TCGGAGCAGC AAACCATACC GAAGTAAGTC GAGGCCNAGG GTTGCTAGTT CCGCTCAATG TACTAGGGGG

5901 ATGTTGTGCA AAAAAGCGGT TAGTCCCTTC GGTCTCCGA TCGTTGTGTC AAGTAAGTTG GCCGCAGTGT TATCACTCAT GGTATGGCA GCACGTGCTA  
TACAAACAGT TTTTTCGCCA ATCGAGGAAG CCAGGAGGCT AGCAACAGTC TTCAATTCAAC CGGCGTCACA ATAGTGAGTA CCAATACCGT CGTGACGTAT

5901

6001 ATTCTCTTAC TGTGATGCCA TCGGTAAGAT GCTTTTCTGT GACTGGTGAG TACTCAACCA AGTCATTCTG AGAATAGTGT ATGCGGCGAC CGAGTTGCTC  
 TAAGAGAATG ACAGTACGGT AGGCATTCTA CGAAAAGACA CTGACCACTC ATGAGTTGGT TCAGTAAGAC TCTTATCACA TAGCGCGCTG GCTCAACGAG

6101 TTGCGCGCGG TCAACACGGG ATAATACCGC GCCACATAGC AGAATTCTAA AAGTGCTCAT CATTCGAAA CCGTCTTCGG GCGCAAACT CTCAAGGATC  
 AACGGGCGCG AGTTGCGCC TATTATGCGG CGGTCTATCG TCTTGAAATT TTCACGAGTA GTRACCTTTT GCAAGAAGCC CCGCTTTTGA GAGTCTCTAG

6201 TTACCGCTGT TGAGATCCAG TTCGATGTAA CCCACTCGTG CACCCAACTG ATCTTCAGCA TCTTTTACTT TCACCAAGCGT TTCTGGGTGA GCAAAAACAG  
 AATGGCGACA ACTCTAGGTC AAGCTACATT GGGTGAGCAC GTGGTTGAC TAGAAGTCGT AGAAAATGAA AGTGGTCGCA AAGACCCACT CGTTTTTGTC

6301 GAAGGCAAAA TGCGGCAAAA AAGGGAATAA GGGCGACACG GAAATGTTGA ATACTCATAC TCTTCTTTT TCAATATTAT TGAAGCATTT ATCAGGGTTA  
 CTTCGGTTTT ACGCGGTTTT TTCCCTTATT CCGCTGTGC CTTACAACT TATGAGTATG AGAAGGAAA AGTTATAATA ACTTCGTAAA TAGTCCCAAT

FIG. 41T

6401 TTTGCTCTCATG AGCGGATACA TATTGGAATG TATTAGAAA AATAACAAA TAGGGTTCC GCGACATTT  
 AACAGAGTAC TCGCCTATGT ATAACTTAC ATAAATCTTT TTATTGTTT ATCCCAAGG CGCGTGAAA

nlaIII  
 rcaI  
 bspHI acII  
 bsmAI bsrBI  
 6401 TTTGCTCTCATG AGCGGATACA TATTGGAATG TATTAGAAA AATAACAAA TAGGGTTCC GCGACATTT  
 AACAGAGTAC TCGCCTATGT ATAACTTAC ATAAATCTTT TTATTGTTT ATCCCAAGG CGCGTGAAA  
 hinPI  
 thal  
 fnuDII/mvni  
 bstOI  
 bsh1236I  
 acII  
 nlaIV hhaI/cfoI  
 maeII  
 hinII/acyI  
 ahaII/bsaHI  
 aatII ddeI  
 CCGCGAAAAG TGGCACCTGA CGTCTAAGAA  
 GGGCTTTTC ACGGTGGACT GCAGATTCTT

sau96I  
 haeIII/palI  
 asuI  
 ecoO109I/draII  
 mnlI  
 bssSI  
 bbsI  
 nlaIII  
 rcaI tru9I  
 bspHI mseI  
 6501 ACCATTATTA TCATGACATT AACCTATAAA AATAGGCGTA TCACGAGGCC CTTTCGTCCTT CAA  
 TGGTAATAAT AGTACTGTAA TTGGATATTT TTATCCGCAT AGTGCTCCG GAAAGCAGAA GTT

FIG. 41U

```

>length: 6563

aatII(GACGTC):      1645 6489
acc65I(GGTACC):      403 823
accI(GTMKAC):      1093 1963 4449
accII(TCCGGA):      3867[dam-]
aci(CCGC):      178 542 805 877 1340 1750 1826 2011 2039 2043 2182 2242 2384 2492 2501 2504
                2628 2781 2784 2787 2906 2926 3005 3045 3094 3141 3226 3241 3309 3342 3367 3412
                3436 3448 3490 3544 3597 3613 3619 3700 3838 3967 3970 3981 4139 4155 4210 4266
                4351 4390 4400 4442 4467 4505 4518 4544 4561 4604 4611 4632 4723 4751 4878 4897
                5018 5128 5263 5272 5634 5725 5916 5962 6083 6127 6204 6313 6412 6459
see hinII
acyI
afIII(ACRYGT):      1307 4678
ageI(ACCGGT):      1788
ahaII/bsaHI(GRCGYC): 1645 1813 2616 2637 2751 3408 6107 6489
ahaII/draI(TTTAAA): 5435 5454 6146
ahdI/eam105I(GACNNNNNGTC): 346 5566
aluI(AGCT):      72 121 252 320 398 532 589 648 1126 1144 1167 1325 1386 1906 2054 2075 2126
                2218 2233 2889 3292 4202 4259 4270 4319 4338 4619 4845 4935 4981 5238 5759 5859
                5922
alw44I/snoI(GTGCAC): 1831 4494 4992 6238
alwI[dam-](GGATC):  412 413 712 713 1171 1471 2578 2579 3300 3870 5245 5319 5331 5416 5429 5893
                6196 6214
alwNI[dcn-](CAGNNNCTG): 1117 1385 5089
apaI(GGGCCC):      1695
apaLI/snoI(GTGCAC): 1831 4494 4992 6238
apoI(RAATY):      1 391 4093
apyI[dcn+](CCWGG): 640 999 1347 1357 1449 1665 1713 1755 1764 2333 3262 3645 4705 4826 4839
aseI/asnI/vspI(ATTAAAT): 5742
asnI
asp700(GAANNNTTC): 905 930 4234 6166
asp718(GGTACC):      403 823
aspHI
aspi
asuI(GGNCC):      1119 1195 1425 1434 1446 1512 1695 1696 1752 2155 2375 2727 3002 3090 3339 3463

```

FIG. 41V



# Stop-Template Primer

SL.97.2      5' CAT GGT ATA GGT TAA ACT TAT TTA CAC 3'

# NNS Randomization Primer

SL.97.3      5' CAT GGT ATA GGT NNS ACT TAT TTA CAC 3'

FIG. 42

Randomization of Position N35 of Variable Light Chain CDR-1  
Amino Acid Frequency

*Phage Display (NNS Codon Library) Sort #3*

Amino Acid	Frequency	% Total	IC50 (nM)
Asparagine (wt)	1	5.6	4.9
Glycine	6	16.6	3.1
Aspartic Acid	3	16.6	3.1
Glutamic Acid	4	22.2	0.1
Alanine	2	5.6	0.2
Lysine	1	5.6	ND
Serine	1	1.9	ND

FIG. 43A

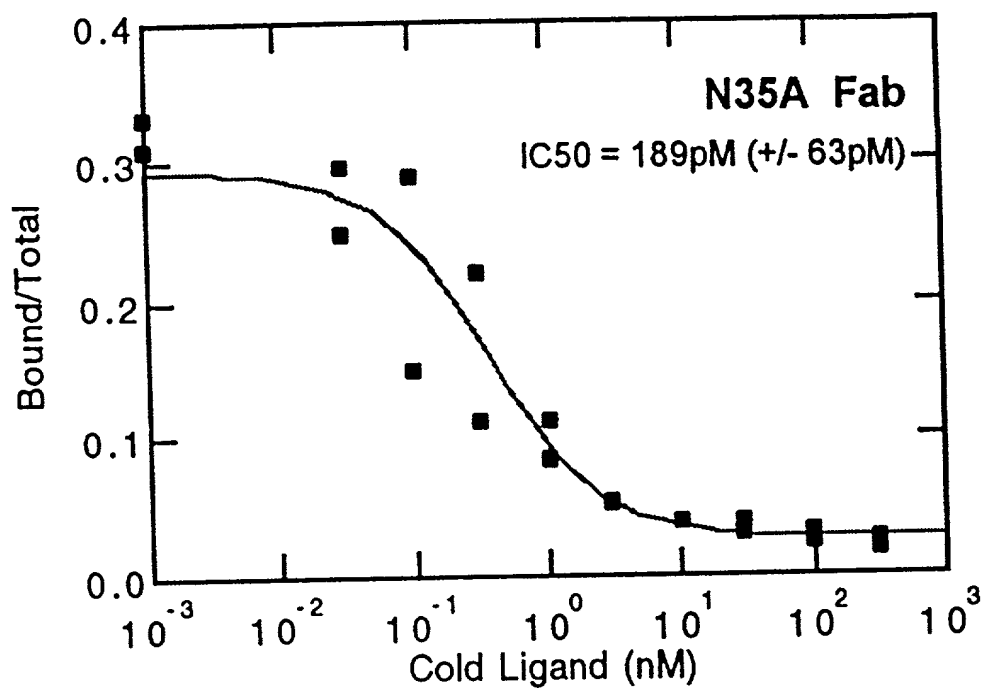


FIG. 43B-1

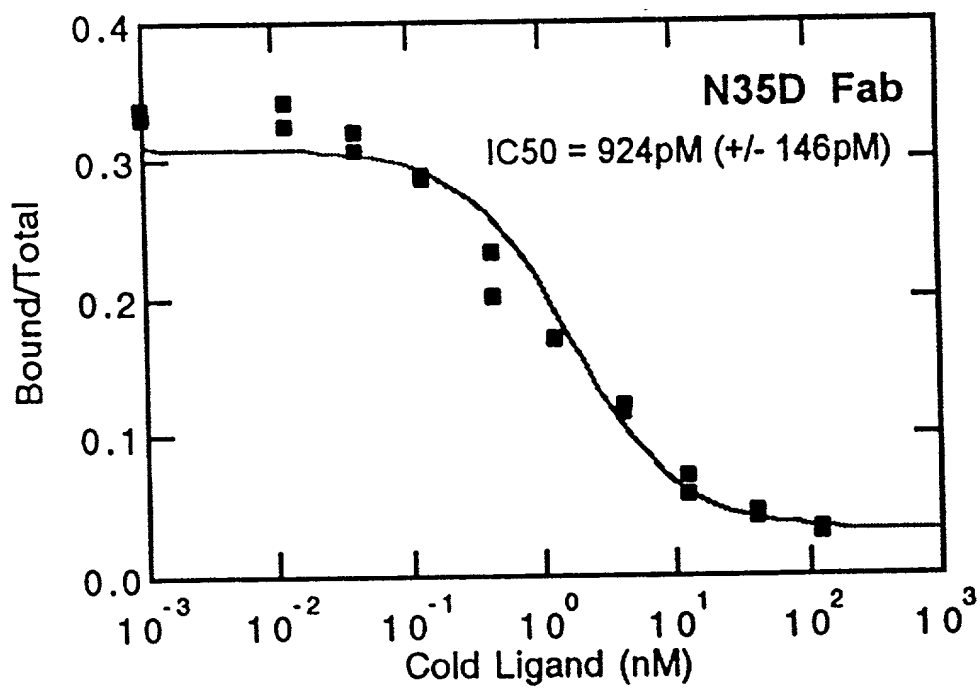


FIG. 43B-2

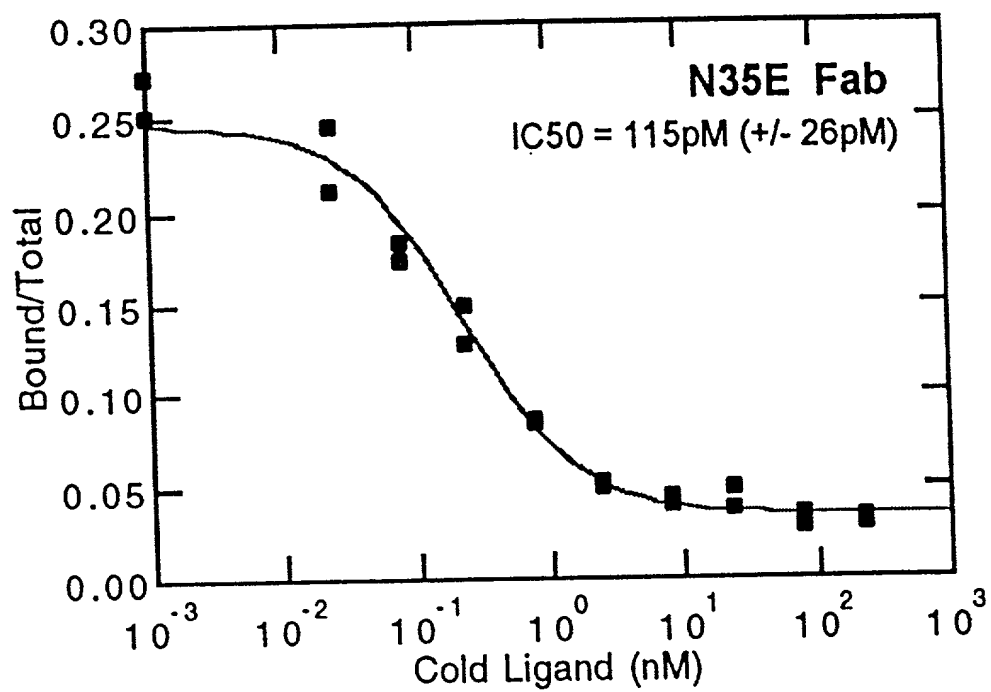


FIG. 43B-3

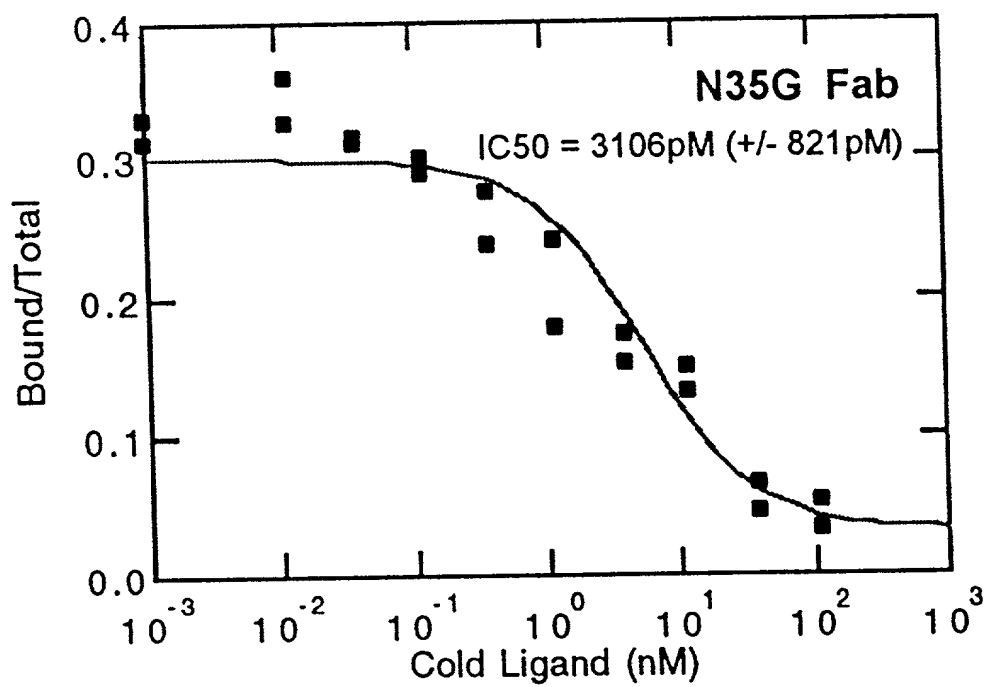
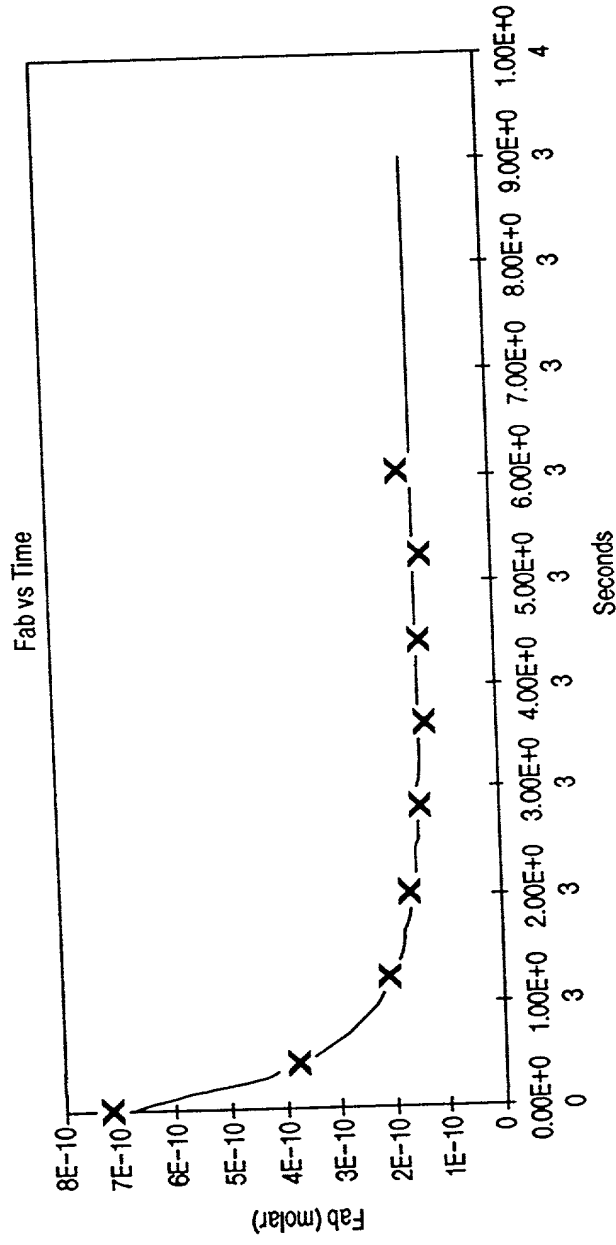


FIG. 43B-4



**Representative Conc versus Time Plot.** Shown is the kinetic data for 6G4V11N35A.F(ab')<sub>2</sub>.

SAMPLE	ka	kd	Kd
6G4V11N35A-Fab	ND	ND	114pM
6G4V11N35A-F(ab') <sub>2</sub>	2.0x10 <sup>6</sup>	2.1x10 <sup>-4</sup>	109pM
6G4V11N35E-Fab	4.7x10 <sup>6</sup>	2.6x10 <sup>-4</sup>	54pM

**FIG. 44**

1 ATGAAAAAGA ATATCGCATT TCTTCTTGCA TCTATGTTTCG TTTTCTCTAT TGCTACAAAC  
 TACTTTTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG  
 -23 M K K N I A F L L A S M F V F S I A T N  
  
 61 GCATACGCTG ATATCCAGAT GACCCAGTCC CCGAGCTCCC TGTCCGCCTC TGTGGGCGAT  
 CGTATGCGAC TATAGGTCTA CTGGGTCAGG GGCTCGAGGG ACAGGCGGAG ACACCCGCTA  
 -3 A Y A D I Q M T Q S P S S L S A S V G D  
  
 121 AGGGTCACCA TCACCTGCAG GTCAAGTCAA AGCTTAGTAC ATGGTATAGG TGAGACGTAT  
 TCCCAGTGGT AGTGGACGTC CAGTTCAGTT TCGAATCATG TACCATATCC ACTCTGCATA  
 18 R V T I T C R S S Q S L V H G I G E T Y  
  
 181 TTACACTGGT ATCAACAGAA ACCAGGAAAA GCTCCGAAAC TACTGATTTA CAAAGTATCC  
 AATGTGACCA TAGTTGTCTT TGGTCTTTT CGAGGCTTTG ATGACTAAAT GTTTCATAGG  
 38 L H W Y Q Q K P G K A P K L L I Y K V S  
  
 241 AATCGATTCT CTGGAGTCCC TTCTCGCTTC TCTGGATCCG GTTCTGGGAC GGATTTCACT  
 TTAGCTAAGA GACCTCAGGG AAGAGCGAAG AGACCTAGGC CAAGACCCTG CCTAAAGTGA  
 58 N R F S G V P S R F S G S G S G T D F T  
  
 301 CTGACCATCA GCAGTCTGCA GCCAGAAGAC TTCGCAACTT ATTACTGTTC ACAGAGTACT  
 GACTGGTAGT CGTCAGACGT CCGTCTTCTG AAGCGTTGAA TAATGACAAG TGTCTCATGA  
 78 L T I S S L Q P E D F A T Y Y C S Q S T  
  
 361 CATGTCCCGC TCACGTTTGG ACAGGGTACC AAGGTGGAGA TCAAACGAAC TGTGGCTGCA  
 GTACAGGGCG AGTGCAAACC TGTCCCATGG TTCCACCTCT AGTTTGCTTG ACACCGACGT  
 98 H V P L T F G Q G T K V E I K R T V A A  
  
 421 CCATCTGTCT TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCTTCTGTT  
 GGTAGACAGA AGTAGAAGGG CGGTAGACTA CTCGTCAACT TTAGACCTTG ACGAAGACAA  
 118 P S V F I F P P S D E Q L K S G T A S V  
  
 481 GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA GGTGGATAAC  
 CACACGGACG ACTTATGAA GATAGGGTCT CTCCGGTTTC ATGTCACCTT CCACCTATTG  
 138 V C L L N N F Y P R E A K V Q W K V D N  
  
 541 GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC AGGACAGCAA GGACAGCACC  
 CGGGAGGTTA GCCCATGAG GGTCTCTCA CAGTGTCTCG TCCTGTCGTT CCTGTCTGTTG  
 158 A L Q S G N S Q E S V T E Q D S K D S T  
  
 601 TACAGCCTCA GCAGCACCCCT GACGCTGAGC AAAGCAGACT ACGAGAAACA CAAAGTCTAC  
 ATGTCGGAGT CGTCGTGGGA CTGCGACTCG TTTCGTCTGA TGCTCTTTGT GTTTCAGATG  
 178 Y S L S S T L T L S K A D Y E K H K V Y  
  
 661 GCCTGCGAAG TCACCCATCA GGGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA  
 CGGACGCTTC AGTGGGTAGT CCCGGACTCG AGCGGGCAGT GTTCTCGAA GTTGTCCCCT  
 198 A C E V T H Q G L S S P V T K S F N R G  
  
 721 GAGTGTAAAG CTGATCCTCT ACGCCGGACG CATCGTGGCC CTAGTACGCA ACTAGTCGTA  
 CTCACAATC GACTAGGAGA TGCGGCCTGC GTAGACCCGG GATCATGCGT TGATCAGCAT  
 218 E C O

FIG. 45

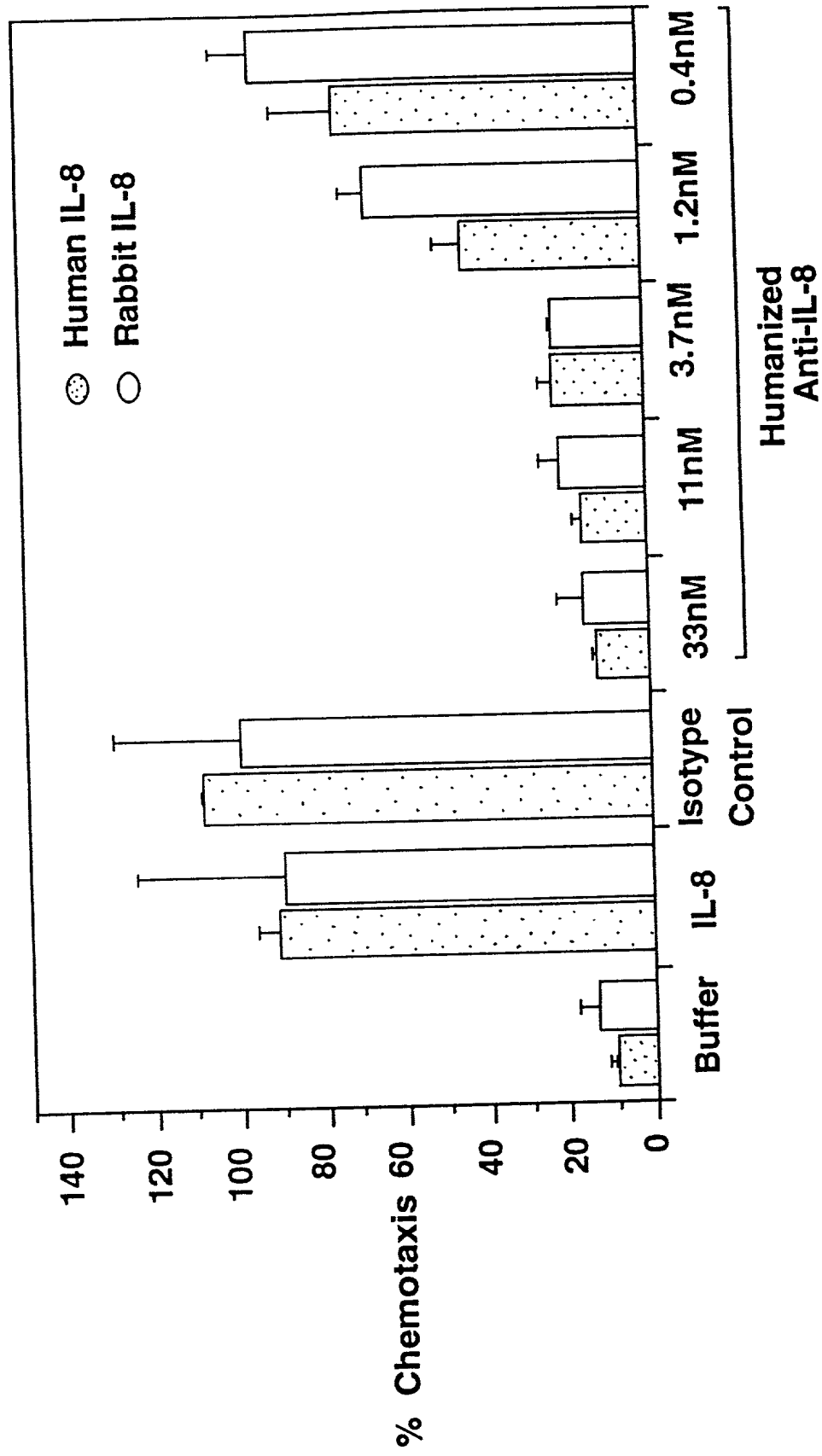


FIG. 46

N35AH1 upr  
5'-CTAGTGCAGTCTGGCGGTGGCCTGGTGCAGCCAGGGGGCTCACTCCGTTTGTCTGTGCAGCTTCTGGCTACTCCTTC-3'

N35AH1 lwr  
5'-TCGAGAAGGAGTAGCCAGAAAGCTGCACAGGACAAACGGAGTGAGCCCCCTGGCTGCACCCAGGCCACCGCCAGACTGCACT  
AG-3'

Bold indicates nucleotide change destroying PvuII site.

FIG. 47



```

> Wed May 7 18:27:36 1997
> /home/ruby/vc/Immbio/afan/ss.p6G425v11.N35A.choSD
> sites: std
> length: 8120 (circular)
> This has the pSVI backbone with the PRK7 cloning linker (pSVI7) and the intron DHFR(ID)
> made from pSVI.WTSD.D by adding a linearization linker(LL) into the HpaI site

```

```

cac8I
alul
sstI
sacI
hgiJII
hgiAI/aspHI
ecII36II
bspl286
bsiHKAI
bmyI
banII
tagI
1 TTCCAGCTCG CCCGACATTG ATTATTGACT AGAGTCGATC GACAGCTGTG GAATGRTGT CAGTTAGGCT GTGGAAAGTC CCCAGGCTCC CCAGCAGGCA
AAGCTCGAGC GGGCTGTAAC TAATACTGA TCTCAGTAG CTCTCGACAC CTTACACACA GTCAATCCCA CACCTTTTCAG GGGTCCGAGG GGTCTCGTCCGT

scrFI mvaI
mboI/ndeII[dam-]
dpoI[dam+]
pvuI/bspCI
pleI dpoII[dam-]
hinfI taqI[dam-]
rmaI mcrI pvuII
maeI bsiEI nspBII
bfaI taqI[dam-]
bsmFI nlaIV cac8I
bsaJI
apyI[dcm+]
bsaJI
sfanI
ppu10I
nsII/avaIII
nlaIII
sphi
nspI
nspHI
cac8I
101 GAAGTATGCA AAGCATGCCAT CTCAATTAGT CAGCAACCCAG GTGTGGAAG GTCCCGAGGT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA
CTTCATACGT TTCTGTACGTA GAGTTAATCA GTCGTTGGTC CACACCTTTC AGGGGTCCGA GGGGTCTGTC

nlaIII
styI
ncoI
bsaJI dsal
aciI bsaJI
201 GTCAGCAACC ATAGTCCCGC CCCTAATCC GCCATCCCG CCCCTAATC CGCCAGTTC CGCCCATCTT CCGCCCATG GCTGACTAAT TTTTTTTATT
CAGTCGTTGG TATCAGGGCG GGGATTGAG CGGGTAGGC GGGGATTAG CGGGTCAAG CGGGGTAAAG GCGGGGGTAC CGACTGATTA AAAAAATAA

```

FIG. 48A

```

fnu4HI      fnu4HI      fnu4HI      fnu4HI      fnu4HI      fnu4HI      fnu4HI      fnu4HI      fnu4HI      fnu4HI
bscFI       bscFI       bscFI       bscFI       bscFI       bscFI       bscFI       bscFI       bscFI       bscFI
bglI        bglI        bglI        bglI        bglI        bglI        bglI        bglI        bglI        bglI
sfiI        sfiI        sfiI        sfiI        sfiI        sfiI        sfiI        sfiI        sfiI        sfiI
haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI
mnlI mnlI mnlI mnlI mnlI mnlI mnlI mnlI mnlI mnlI
haeIII/palI bsaJI bsaJI bsaJI bsaJI bsaJI bsaJI bsaJI bsaJI bsaJI
mnlI bsaJI acII haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI
301 TATGCAGAGG CCGAGGCCG CTGCGCTCT GAGCTATTCC AGAAGTAGTG AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG CAAAAGCTA GCTTATCCGG
ATACGTCTCC GGCTCCGGC GAGCGGAGA CTCGATAAGG TCTTCATCAC TCCTCCGAAA AAACCTCCG ATCCGAAAAC GTTTTTCGAT CGAATAGGCC

tflI        tflI        tflI        tflI        tflI        tflI        tflI        tflI        tflI        tflI
hinFI       hinFI       hinFI       hinFI       hinFI       hinFI       hinFI       hinFI       hinFI       hinFI
acII        acII        acII        acII        acII        acII        acII        acII        acII        acII
thai        thai        thai        thai        thai        thai        thai        thai        thai        thai
fnuDII/mvni fnuDII/mvni fnuDII/mvni fnuDII/mvni fnuDII/mvni fnuDII/mvni fnuDII/mvni fnuDII/mvni fnuDII/mvni fnuDII/mvni
bstUI       bstUI       bstUI       bstUI       bstUI       bstUI       bstUI       bstUI       bstUI       bstUI
bsh1236I    bsh1236I    bsh1236I    bsh1236I    bsh1236I    bsh1236I    bsh1236I    bsh1236I    bsh1236I    bsh1236I
CGGGGAACGG TGCATTGGAA CGCGGATTCC CCGTGCCAAAG AGTGAGGTAA GTACCGCTTA TAGAGCGATA ACAGGATTTT ATCCCGCTG CCATCATGGT
401 GCGCCTTGCC ACGTAACCTT GCGCCTAAGG GGCACGGTTC TCACTGCATT CATGGCGGAT ATCTCGCTAT TCTCCTAAA TAGGGGGAC GGTAGTACCA
DHFH ATG~

haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI haeIII/palI
haeI        haeI        haeI        haeI        haeI        haeI        haeI        haeI        haeI        haeI
scrFI       scrFI       scrFI       scrFI       scrFI       scrFI       scrFI       scrFI       scrFI       scrFI
mval        mval        mval        mval        mval        mval        mval        mval        mval        mval
ecorII      ecorII      ecorII      ecorII      ecorII      ecorII      ecorII      ecorII      ecorII      ecorII
dsav        dsav        dsav        dsav        dsav        dsav        dsav        dsav        dsav        dsav
bstNI       bstNI       bstNI       bstNI       bstNI       bstNI       bstNI       bstNI       bstNI       bstNI
acyI[dm+]   acyI[dm+]   acyI[dm+]   acyI[dm+]   acyI[dm+]   acyI[dm+]   acyI[dm+]   acyI[dm+]   acyI[dm+]   acyI[dm+]
bsaI        bsaI        bsaI        bsaI        bsaI        bsaI        bsaI        bsaI        bsaI        bsaI
bsaI        bsaI        bsaI        bsaI        bsaI        bsaI        bsaI        bsaI        bsaI        bsaI
pflMI       pflMI       pflMI       pflMI       pflMI       pflMI       pflMI       pflMI       pflMI       pflMI
bslI        bslI        bslI        bslI        bslI        bslI        bslI        bslI        bslI        bslI
bsmFI       bsmFI       bsmFI       bsmFI       bsmFI       bsmFI       bsmFI       bsmFI       bsmFI       bsmFI
sfNI        sfNI        sfNI        sfNI        sfNI        sfNI        sfNI        sfNI        sfNI        sfNI
tagI        tagI        tagI        tagI        tagI        tagI        tagI        tagI        tagI        tagI
501 TCGACCATG AACTGCATCG TCGCCGTGTC CCAAAATATG GGGATTGGCA AGAAGCGAGA CCTACCCCTGG CCTCGCTCA GGAACGAGTT CAAGTACTTC
ACGTGGTAAC TTGACGTAGC AGCGGCACAG GTTTTTATAC CCTAACCGT TCTTGCCTCT GGATGGGACC GGAGCGAGT CCTTGCTCAA GTTCATGAAG

```

FIG. 48B

601 CAAAGATGA CCACACCTC TTCAGTGGAA GGTAAACAGA ATCTGTGAT TATGGGTAGG AAAACCTGGT TCTCCATTCC TGAGAGAAAT CGACCTTTAA  
 GTTTCTTACT GGTGTTGGAG AAGTCACCTT CCATTGTCT TAGACCACTA ATACCCATCC TTTTGGACCA AGAGGTAAGG ACTCTTCTTA GCTGGAAT

601 CAAAGATGA CCACACCTC TTCAGTGGAA GGTAAACAGA ATCTGTGAT TATGGGTAGG AAAACCTGGT TCTCCATTCC TGAGAGAAAT CGACCTTTAA  
 GTTTCTTACT GGTGTTGGAG AAGTCACCTT CCATTGTCT TAGACCACTA ATACCCATCC TTTTGGACCA AGAGGTAAGG ACTCTTCTTA GCTGGAAT

sstI  
 sacI  
 hgiJII  
 hgiAI/aspHI  
 ecl136II  
 bsp1286  
 bslHKAI  
 bmyI

tru9I  
 mseI  
 aseI/asnI/vspI  
 701 AGGACAGAT TAATATAGTT CTCAGTAGAG AACTCAAAGA ACCACCAGA GGAGTTCATT TTCTTGCCAA AAGTTGGAT GATGCCTTAA GACTTATTGA  
 TCTGTCTTA ATTATATCA GAGTCATCTC TTGAGTTTCT TGGTGTGCT CCTCGAGTAA AAGAAGGTT TTCAAACCTA CTACGGAAT CTGAATAACT

tru9I  
 aflII/bfRI  
 bstXI  
 foki sfaNI mseI  
 haeIII/palI  
 haeI

801 ACAACCGGA TTGGCAAGTA AAGTAGACAT GGTGAGGATA GTCGGAGGCA GTTCTGTTTA CCAGGAAGCC ATGAATCAAC CAGGCCACCT TAGACTCTTT  
 TGTGGCCTT AACCGTTTAT TTCATCTGTA CCAACCTAT CAGCCTCCGT CAAGACAAAT GGTCTTCG TACTTAGTTG GTCCGGTGA ATCTGAGAA

scrFI  
 mvaI  
 ecorII  
 dsav  
 bstNI  
 apyI[dcM+]  
 sexAI  
 tfiI  
 hinfI  
 ddeI mboII taqI  
 ahaII/draI  
 mseI  
 scrFI  
 mvaI  
 ecorII  
 dsav  
 bstNI  
 apyI[dcM+]  
 hinfI  
 ddeI pleI  
 hinfI

FIG. 48C

901 GTGACAAGGA TCATGCAGGA ATTTGAAAGT GACACGTTTT TCCAGAGAAAT TGATTTGGGG AAATATAAAC CTCCTCCAGA ATACCCAGGC GTCCTCTCTG  
 CACTGTTTCT AGTAGCTCT TAACTTTCA CTGTCAAAA AGGCTCTTA ACTAAACCCC TTTATATTG GAGAGGTCT TATGGGTCCG CAGGAGAGAC

```

          hgaI
          hinII/acyI
          ahaII/bsaHI
scrFI
mvaI      mnII
ecoRII
dsav
bstNI     ecoNI
          apyI[dcM+] mnII
          bsaJI     bali ddel
          mnII
          901 GTGACAAGGA TCATGCAGGA ATTTGAAAGT GACACGTTTT TCCAGAGAAAT TGATTTGGGG AAATATAAAC CTCCTCCAGA ATACCCAGGC GTCCTCTCTG
          CACTGTTTCT AGTAGCTCT TAACTTTCA CTGTCAAAA AGGCTCTTA ACTAAACCCC TTTATATTG GAGAGGTCT TATGGGTCCG CAGGAGAGAC

```

```

scrFI
mvaI
ecoRII
dsav
bstNI
          apyI[dcM+]
sau96I
avaII
          1001 AGGTCCAGGA GGAAGAGGC ATCAAGTATA AGTTTGAAGT CTACGAGAG AAAGACTAAC AGGAAGATCC TTTCAGATT TCTGCTCCG TCCTAAAGCT
          TCCAGGTCCT CCTTTTCCG TAGTTCATAT TCAACTTCA GATGCTCTTC TTTCTGATTG TCCTTCTAGG AAAGTTCAAG AGACGAGGG AGGATTTCGA
          ^END DHFR

```

```

          sfanI
          mboII
          mnII
          1001 AGGTCCAGGA GGAAGAGGC ATCAAGTATA AGTTTGAAGT CTACGAGAG AAAGACTAAC AGGAAGATCC TTTCAGATT TCTGCTCCG TCCTAAAGCT
          TCCAGGTCCT CCTTTTCCG TAGTTCATAT TCAACTTCA GATGCTCTTC TTTCTGATTG TCCTTCTAGG AAAGTTCAAG AGACGAGGG AGGATTTCGA
          ^END DHFR

```

```

          styI
          bsaJI
          sau3AI
          mboI/ndeII[dam-]
          aluI
          fnu4HI
          bsoFI
          bsvI
          asei/asnI/vspI
          1101 ATGCATTTT ATAAGACCAT GGGACTTTTG CTGGCTTTAG ATCCCTTGG CTTCGTTAGA ACGCAGCTAC AATTAATACA TAACCTTATG TATCATACAC
          TACGTAAAAA TATTCTGGTA CCTGTAAAC GACCGAATC TAGGGGAACC GAAGCAATCT TGGCTCGATG TTAATTATGT ATTGGAATAC ATAGTATGTG

```

```

sau96I
avaII
asuI
scrFI
mvaI
ecoRII

```

FIG. 48D

1201 ATACGATTTA GGTGACACTA TAGATAACAT CCACCTTTGCC TTTCTCTCCA CAGGTGTCCA bslI bsaJI foki maeIII hphi scfI  
TATGCTAAAT CCACTGTGAT ATCTATTGTA GGTGAACCG AAGAGAGAGGT GTCCACAGGT bsaJI bsaJI apyI[dcM+] mnlI bspDI[dam-]  
seq from PRK6425VH: Cla-AvII^

1301 CCACCATGGG ATGGTCATGT ATCATCCTTT TTCTAGTAGC AACTGCAACT GGAGTACATT CAGAAGTTCA GCTAGTGCAG TCTGGCGGTG GCCTGGTGCA  
GGTGTACCC TACCAGTACA TAGTAGGAAA AAGATCATCG TTGACGTTGA CCTCATGTAA GTCTTCAAGT CGATCAGTC AGACGCCAC CGGACCCAGT  
E V Q L V Q S G G L V Q

1401 CGGTCCCCG AGTGAGGCNA ACAGGACACG TCGAAGACCG ATGAGGACCG TACTCCTTCT CGAGTCACTA TATGCACTGG GTCCGTCAGG CCCCGGGTAA GGGCCTGGAA  
CGGTCCCCG AGTGAGGCNA ACAGGACACG TCGAAGACCG ATGAGGACCG TACTCCTTCT CGAGTCACTA TATGCACTGG GTCCGTCAGG GTCCGTCAGG CCCCGGGTAA GGGCCTGGAA  
CGGTCCCCG AGTGAGGCNA ACAGGACACG TCGAAGACCG ATGAGGACCG TACTCCTTCT CGAGTCACTA TATGCACTGG GTCCGTCAGG GTCCGTCAGG CCCCGGGTAA GGGCCTGGAA

14 P G G S L R L S C A A S G Y S F S S H Y M H W V R Q A P G K G L E

FIG. 48E

bsII  
sau3AI  
mboI/ndeI[dam-]  
dpmI[dam+]  
dpmII[dam-]  
alwI[dam-] hphI  
1501 TGGGTTCGAT ATATTGATCC, TTCCAATGGT GAAACTACGT ATAATCAAAA GTTCAAGGGC CGTTTCACTT TATCTCGGA CAACCTCCAAA AACACAGCAT  
ACCCAACCTA TATAACTAGG AAGGTTACCA CTTTGATGCA TATTAGTTT CAAGTTCCCG GCAAGTGAA ATAGAGCGCT GTTGAGGTTT TTGTGTCGTA  
47 W V G Y I D P S N G E T T Y N Q K F K G R F T L S R D N S K N T A Y  
hinII/acyI  
ahaiI/bsaHI  
aatII  
bsrI  
maeIII  
hphI  
mboII  
taqI  
3cfI  
pstI  
bsgI  
bpmI  
cac8I ddeI drdI  
cac8I  
1601 ACCTGCAGAT GAAACGCTG CGTGTGAGG ACACTGCCGT CTATTACTGT GCAAGGGG ATTATCGCTA CAATGGTGAC TGGTCTTCG ACCTCTGGG  
TGACGCTCTA CTTGTGGAC GCACGACTCC TGTGACGGCA GATAATGACA CGTTCTCCC TAATAGCGAT GTTACCACGT ACCAAGAAGC TGACAGCCCC  
81 L Q M N S L R A E D T A V Y C A R G D Y R Y N G D W F F D V W G  
scrFI  
mvaI  
ecoRII  
dcaV  
bsaJI  
sau96I  
haeIII/palI  
asul  
fnu4HI  
bscFI  
bsp1286  
acII  
bsaJI  
bpyI  
nspBII  
apyI[dcm+]  
1701 TCAAGGAACC CTGGTCACCG TCTCCTCGC CTCACCAAG GGCACATCG TCITCCCCCT GGCACCTCC TCCAAGGACA CCTCTGGGG CACAGCGGCC  
AGTTCCCTGG GACCACTGGC AGAGAGCCG GAGTGTGTT CCGGTAGCC AGAAGGGGA CCGTGGAGG AGTTTCTCT TCAAGCCCC GTGTCCGGG  
114 Q G T L V T V S S A S T K G P S V F P L A P S S K S T S G G T A A

FIG. 48F

[illegible]

FIG. 48G

[illegible]

FIG. 48H







3001 GAATGTGTGT CAGTTAGGT GTGGAAAGTC CCGAGGCTCC CCAGCAGGCA GAAGTATGCA AAGCATGCAT CTCAATTAGT CAGCAACCAG GTGTGGAAAG  
CTTACACACA GTCAATCCCA CACCTTTCAG GGGTCCGAGG GGTCGTCCGT CTTCATACGT TTCGTACGTA GAGTTAATCA GTCGTTGGTC CACACCTTTC

3001 GAATGTGTGT CAGTTAGGT GTGGAAAGTC CCGAGGCTCC CCAGCAGGCA GAAGTATGCA AAGCATGCAT CTCAATTAGT CAGCAACCAG GTGTGGAAAG  
CTTACACACA GTCAATCCCA CACCTTTCAG GGGTCCGAGG GGTCGTCCGT CTTCATACGT TTCGTACGTA GAGTTAATCA GTCGTTGGTC CACACCTTTC

3101 TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA GTCAGCAACC ATAGTCCGC CCCTAACTCC GCCATCCG CCCCTAACTC  
AGGGGTCCGA GGGTCCGTC CAGTTCATAC GTCTTCATAC GTTTCGTACG TAGAGTTAAT CAGTCGTGG TATCAGGGCG GGGATTGAG GGGATTGAG

3201 CGCCAGTTC CGCCATTCT CGGCCCATG GCTGACTAAT TTTTATTAT TATGCAGAG CCGAGGCCG CTGCGCTCT GAGCTATTCC AGAAGTAGTG  
GCGGTCAAG GCGGTAAGA GCGGGGTAC CGACTGATTA AAAAAATAA ATACGTCCTC GGCTCCGGC GAGCCGGAGA CTCGATAAGG TCTTCATCAC

FIG. 48K





[illegible]

**FIG. 48N**



4401 TTTTTCACCTG CATTCCTAGTT GTGGTTTGTC CAAACTCATC AATGTATCTT ATCATGTCTG GATCGATCGG GAATTAATTC GCGCAGCAC CATGCGCTGA  
 4501 AATAACCTCT GAAAGAGGAA CTGGGTTAGG TACCTTCTGA GCGGAAAGA ACCAGCTGTG GAATGTGTGT CAGTTAGGGT GTGGAAAGTC CCCAGGCTCC  
 TTAATTGGAGA CTTTCTCCTT GAACCAATCC ATGGAAGACT CCGCTTTCT TGGTCGACAC CTACACACA GTCAATCCCA CACCTTTCAG GGGTCCGAGG

4401 TTTTTCACCTG CATTCCTAGTT GTGGTTTGTC CAAACTCATC AATGTATCTT ATCATGTCTG GATCGATCGG GAATTAATTC GCGCAGCAC CATGCGCTGA  
 4501 AATAACCTCT GAAAGAGGAA CTGGGTTAGG TACCTTCTGA GCGGAAAGA ACCAGCTGTG GAATGTGTGT CAGTTAGGGT GTGGAAAGTC CCCAGGCTCC  
 TTAATTGGAGA CTTTCTCCTT GAACCAATCC ATGGAAGACT CCGCTTTCT TGGTCGACAC CTACACACA GTCAATCCCA CACCTTTCAG GGGTCCGAGG

rsal  
 csp6I  
 nlaIV  
 kpnI  
 hgiCI  
 banI  
 asp718  
 acc65I  
 mnlI  
 ddeI  
 actI  
 nspBII  
 pvuII  
 nspBII  
 aluI  
 pvuII  
 nspBII  
 scrFI  
 mvaI  
 ecorII  
 dsav  
 bstNI  
 apyI[dcmt+]  
 bsaJI  
 bsmFI  
 nlaIV

4401 TTTTTCACCTG CATTCCTAGTT GTGGTTTGTC CAAACTCATC AATGTATCTT ATCATGTCTG GATCGATCGG GAATTAATTC GCGCAGCAC CATGCGCTGA  
 4501 AATAACCTCT GAAAGAGGAA CTGGGTTAGG TACCTTCTGA GCGGAAAGA ACCAGCTGTG GAATGTGTGT CAGTTAGGGT GTGGAAAGTC CCCAGGCTCC  
 TTAATTGGAGA CTTTCTCCTT GAACCAATCC ATGGAAGACT CCGCTTTCT TGGTCGACAC CTACACACA GTCAATCCCA CACCTTTCAG GGGTCCGAGG

4401 TTTTTCACCTG CATTCCTAGTT GTGGTTTGTC CAAACTCATC AATGTATCTT ATCATGTCTG GATCGATCGG GAATTAATTC GCGCAGCAC CATGCGCTGA  
 4501 AATAACCTCT GAAAGAGGAA CTGGGTTAGG TACCTTCTGA GCGGAAAGA ACCAGCTGTG GAATGTGTGT CAGTTAGGGT GTGGAAAGTC CCCAGGCTCC  
 TTAATTGGAGA CTTTCTCCTT GAACCAATCC ATGGAAGACT CCGCTTTCT TGGTCGACAC CTACACACA GTCAATCCCA CACCTTTCAG GGGTCCGAGG

FIG. 48P



[illegible]

FIG. 48Q

5001 TGGCGTTACC CAACCTTAATC GCCTTGACG ACATCCCGCC TTGCGCCAGCT GCGGTAATAG CGGCAATATC GCTTCTCCG GCGTGGCTAG CGGGAAGGT TGTCAACGCA

5001 TGGCGTTACC CAACCTTAATC GCCTTGACG ACATCCCGCC TTGCGCCAGCT GCGGTAATAG CGGCAATATC GCTTCTCCG GCGTGGCTAG CGGGAAGGT TGTCAACGCA

5101 AGCCTGAATG CGGAATGGCG CCTGATGCGG TATTTCTCC TTACGCATCT GTGCGGTATT TCACACCGCA TAGTCAAG CAACCATAGT ACGGCCCCCTG

5201 TAGCGGGCGCA TTAAGCGCGG CGGGTGTGGT GGTACGCGC AGCGTGACCG CTACACTTGC CAGCGCCCTA GCGCCGCTC CTTTCGCTTT CTTCCCTTCC

FIG. 48R







hinPI hpaII mspI  
 hhaI/cfoI hpaII  
 mstI scrFI  
 aviII/fspI nciI tru9I  
 maeII dsaV msel  
 psp1406I cauII asel/asnI/vspI  
 6601 CGACGAGCGT GACACACGCA TGCCAGCAGC AATGGCAACA AACTATTAACT TGGCGAAGCTA CTTACTCTAG CTTCGCCGCA ACAATTAAATA  
 GCTGCTCGCA CTGTGTGCT ACGGTCGTGCT TTACCGTGTG TGCACACGCGT TTGATAATTG ACCGCTTGAT GAATGAGATC GAAGGCGCGT TGTAAATTAT  
 bglI mspI  
 sau96I hpaII  
 haeIII/palI cfr10I/bsrFI  
 hinPI asuI mspI nlaIV hphI bsmAI  
 hhaI/cfoI hpaII bpmI/gauI/dcm-] bsaI  
 6701 GACTGGATGG AGCGGATAA AGTTGCAGCA CCACCTTCTC GCTCGGCCCT TCCGGCTGGC TGGTTATTG CTGATAAATC TGGAGCGGT GAGCGTGGT  
 CTGACCTACC TCCGCCTATT TCAACGTCCT GGTGAAGACG CGAGCGGGGA AGCCGACCG ACCAATAAC GACTATTATG ACCTCGGCCA CTGCGACCCA  
 aciI  
 fnu4HI haeIII/palI pleI  
 fnuDII/mvnI bsoFI sau96I hinfi  
 bstUI nlaIV foki  
 bsh1236I bsrDI bsrI asuI mnli  
 6801 CTGCGGGTAT CATTCAGCA CTGGGCGCAG ATGGTAAGCC CTCCCGTATC GTAGTTATCT ACACGACGGG GAGTCAGGCA ACTATGGATG AACCAATAG  
 GAGCGGCATA GTACGTCGT GACCCGGTC TACCATTGG GAGGCGATAG CATCAATAGA TGTGCTGCC CTGAGTCCGT TGATACCTAC TTGCTTTATC  
 ddeI  
 sau3AI nlaIV tru9I  
 mboI/ndeII[dam-] msel  
 dpnI[dam+] hgiCI ahaIII/draI msel  
 dpnII[dam-] bani mnli maeIII  
 6901 ACAGATCGCT GAGATAGGTG COTCAGTAT TACCATTTGG TAACTGTGAG ACCAAGTTTA CTCATATATA CTTAGATTG ATTTAAACT TCATTTTAA  
 TGTCTAGCGA CTCTATCCAC GGAGTGACTA ATTGCTAACC ATTGACAGTC TGTTCAAT GAGTATATAT GAAATCTAAC TAAATTTTGA AGTAAATAAT  
 rmaI sau3AI  
 maeI mboI/ndeII[dam-]  
 sau3AI hphI mboI/ndeII[dam-]  
 mboI/ndeII[dam-] dpnI[dam+] msel  
 dpnII[dam-] dpnII[dam-] dpnII[dam-]  
 tru9I bstYI/xhoII bstYI/xhoII nlaIII hgaI  
 msel alwi[dam-] bstYI/xhoII rcaI ddeI  
 ahaIII/draI bfaI mboII[dam-] maeII  
 7001 TTTTAAAGGA TCTAGGTGAA GATCCTTTT GATAATCTCA TGACCAAAAT CCCTTAACGT GAGTTTGGT TCCACTGAGC GTCAGACCCC GTAGAAAGA  
 AAATTTTCT AGATCCACTT CTAGGAAAAA CTATTAGAGT ACTGGTTTGA GGAATTGCA CTCAAAAGCA AGGTGACTCG CAGTCTGGG CATCTTTCT

FIG. 48V

```

sau3AI
mboII[dam-]
sau3AI mboI/ndeII[dam-] thaI
mboI/ndeII[dam-] fnuDII/mvNI
dpnI[dam+] dpnI[dam+] bstUI cac8I
dpnII[dam-] dpnII[dam-] bsh1236I fnu4HI
bstYI/xhoII alwI[dam-] hinPI bsoFI
alwI[dam-] bstYI/xhoII hhaI/cfoI bbvI
7101 TCAAAGGATC TTCTTGAGAT CCTTTTTC TGCGGTAAT TGCGGTAAT CACGACGGAAC GTTGTGTTT TGGTGGCGA AACCACCGCT ACCAGCGGTG GTTGTGTTGC CGGATCAAGA
AGTTTCCTAG AAGAAGCTA GGAAGGAGG ACGGCAATTA ACGGCAATTA ACGGCAATTA ACGGCAATTA ACGGCAATTA ACGGCAATTA ACGGCAATTA ACGGCAATTA ACGGCAATTA ACGGCAATTA
bsrI hinPI rmaI haeIII/palI
maeIII eco57I hhaI/cfoI maeI haeI
7201 GCTACCAACT CTTTTTCCGA AGTAACTGG CTTCAGCAGA GCGCAGATAC CAAATACTGT CCTTCTAGTG TAGCCGTAGT TAGGCCACCA CTTCAGAGAC
CGATGGTTGA GAAAAGGCT TCCATTGACC GAAGTCGTCT CGGCTCTATG GTTTATGACA GGAAGATCAC ATCGGCATCA ATCGGCTGGT GAAGTTCTTG
fnu4HI scrFI
bsoFI nciI
bbvI mspI
fnu4HI alwNI[dcM-] hpaII pleI
bsrI bsoFI bsrI cauII hinfI
maeIII bbvI bsrI
7301 TCTGTAGCAC CGCCTACATA CCTCGCTCTG CTAATCCTGT TACCAGTGGC TGCTGCCAGT GGCATTAAGT CGTGTCTTAC CGGGTTGGAC TCAAGACCGAT
AGACATCGTG GCGGATGTAT GGAGCGAGAC GATTAGGACA ATGGTCACCG ACGACGGTCA CGGCTATTCA GCACAGATG GCCCAACCTG AGTTCTGCTA
acII hgiAI/aspHI
nspBII bsp1286
fnu4HI bsiHKAI
bsoFI bmyI
bbvI mcrI apaLI/snoI
hinPI bsiEI alw44I/snoI aluI
hhaI/cfoI
7401 AGTTACCGGA TAAGCGCAG CGTTCGGGT GAACGGGGG TTCTGTCACA CAGCCAGCT TGGAGCGAAC GACCTACACC GAACCTGAGAT ACCTACAGCG
TCAATGGCCT ATTCCGGCTC GCCAGCCCGA CTTGCCCCCGA AAGCAGCTGT GTTCGGTCCA ACCTCGCTTG CTGGATGCG CTTGACTCTA TGGATGTCGC

```

FIG. 48W





```

    thaI
    fnuDII/mvnI
    bstUI
    bshI236I
    hinPI
    hhaI/cfoI
    thaI
    fnuDII/mvnI          cac8I
    bstUI haeIII/palI    aluI
    bshI236I             tru9I pvuII
    bslI eaeI tfII aseI/asnI/vsPI
    aciI cfrI hinFI mseI nspBII
    7901 CCGCCTCTCC CCGCGCGTTG GCCGATTTCAT TAATCCAGCT GGACAGCACAG GTTTCCTCCGAC TGGAAAGCGG GCAGTGAGCG CAACGCAATT AATGTGAGTT
        mnII
        aciI
        GCGCGAGAGG GCGCGGCAAC CGGCTAAGTA ATTAGGTGCA CCGTGCTGTC CAAAGGGCTG ACCTTTCGCC CGTCACCTGC GTTGGGTTAA TTACACTCAA
        tru9I mseI maeIII
        hinPI hhaI/cfoI aseI/asnI/vsPI
        cac8I aciI bsrI
        bsrI TGGAAAGCGG GCAGTGAGCG CAACGCAATT AATGTGAGTT
        mspl hpaII
        nlaIV bstNI
        hgiCI apyI(dcm+)
        bani bsaJI
        8001 ACCTCACTCA TTAGGCACCC CAGGCTTTAC ACTTTATGCT TCCGGCTCGT ATGTTGTGTG GAATTGTGTG CCGATAACAA TTTCACACAG GAAACAGCTA
        mnII
        TGGAGTGAGT AATCCGTGGG GTCCGGAATG TGAATATACGA AGGCGGAGCA TACAACACAC CTTAACACTC GCCTATTGTT AAAGTGTGTC CTTTGTGCGAT
        aciI aluI

```

FIG. 48Y

```

tru9I
mseI
asel/asnI/vspI
xmnl
nlaIII asp700
8101 TGACCATGAT TACGAATTAA
      ACTGGTACTA ATGCTTAATT

>length: 8120

aatII(GACGTC): 1690 5947
acc65I(GGTACC): 2969 3967 4529
accI(GTMKAC): 823 1039 2738 4237
aciI(CCGC): 217 229 238 250 260 271 317 422 454 485 574 1385 1795 1871 2248 2250 2758 2982
              3167 3179 3188 3200 3210 3221 3267 3372 3404 3449 3686 3949 4021 4318 4542 4727
              4739 4748 4760 4770 4781 4827 4910 4914 5070 5127 5153 5166 5203 5217 5220 5248
              5275 5680 5699 5741 5751 5790 5979 6026 6125 6234 6311 6355 6476 6522 6713 6804
              7166 7175 7310 7420 7541 7560 7687 7715 7806 7827 7834 7877 7901 7911 7967 8070
see hinlI
acyI
aflII/bfII(CTTAAG): 786
aflIII(ACRYGT): 932 7758
ageI(ACCGGT): 1833
ahaII/psaHI(GRCGYC): 988 1690 1858 5117 5947 6329
ahaII/draI(TTTAA): 696 4935 6290 6982 7001
ahdI/eam1105I(GACNNNNNGTC): 2087 6865
alul(AGCT): 5 44 332 386 390 753 1097 1165 1370 1431 1951 2603 2751 2784 3282 3336 3340
              3562 3566 3676 3733 3792 4270 4288 4311 4344 4554 4842 4896 4954 5047 5333 5590
              5803 5822 6516 6579 6679 7200 7457 7593 7819 7937 8096
alw44I/snoI(GTGCAC): 1876 5651 6198 7444

```

FIG. 48Z

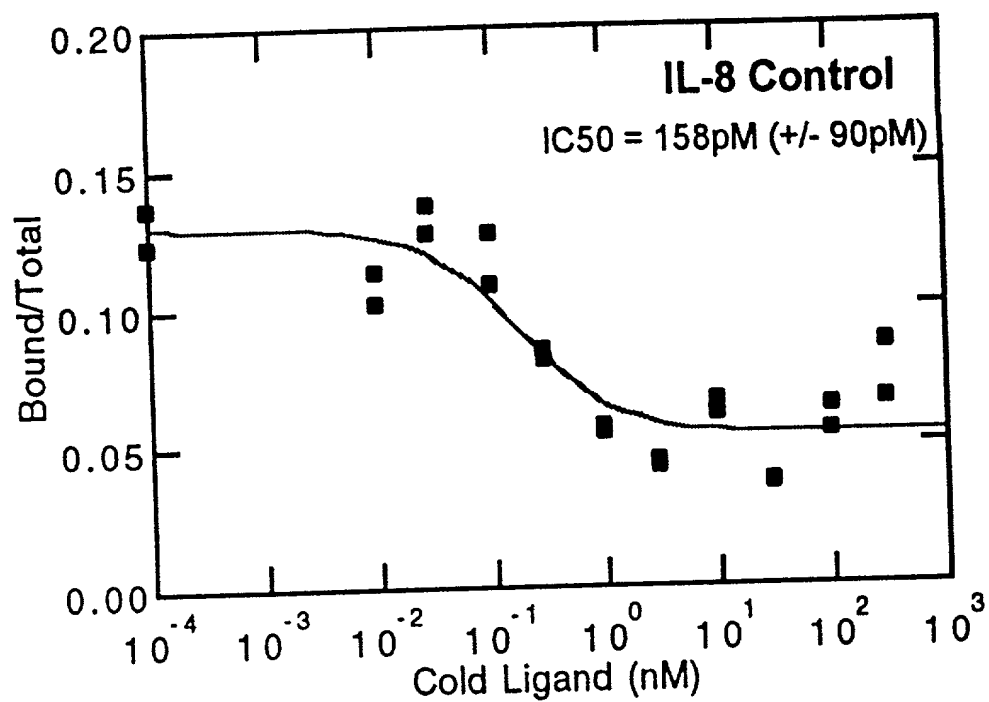


FIG. 49A

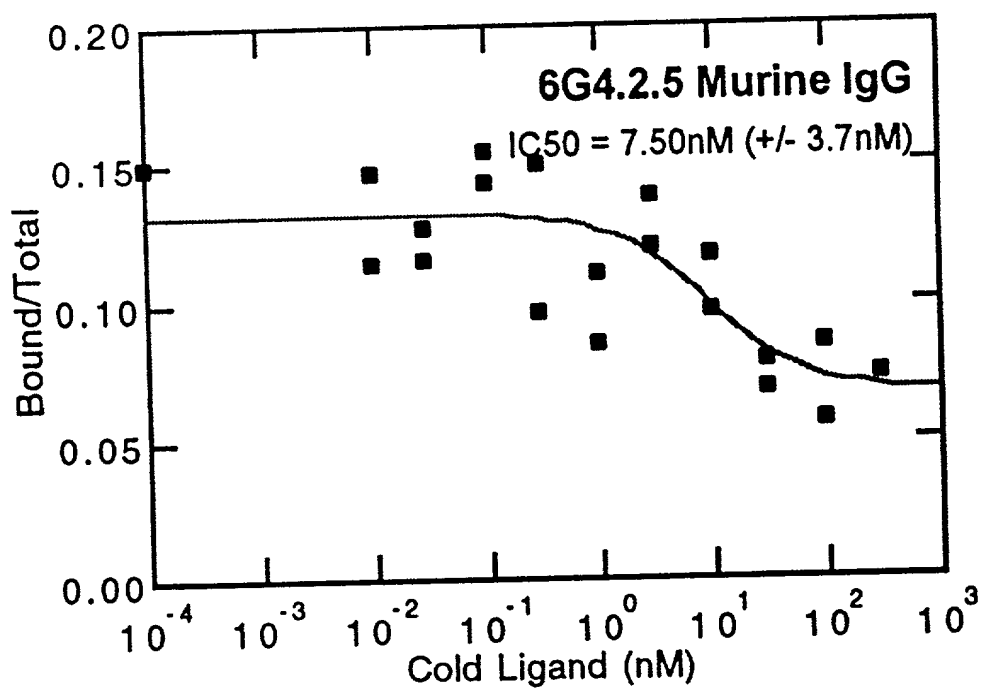
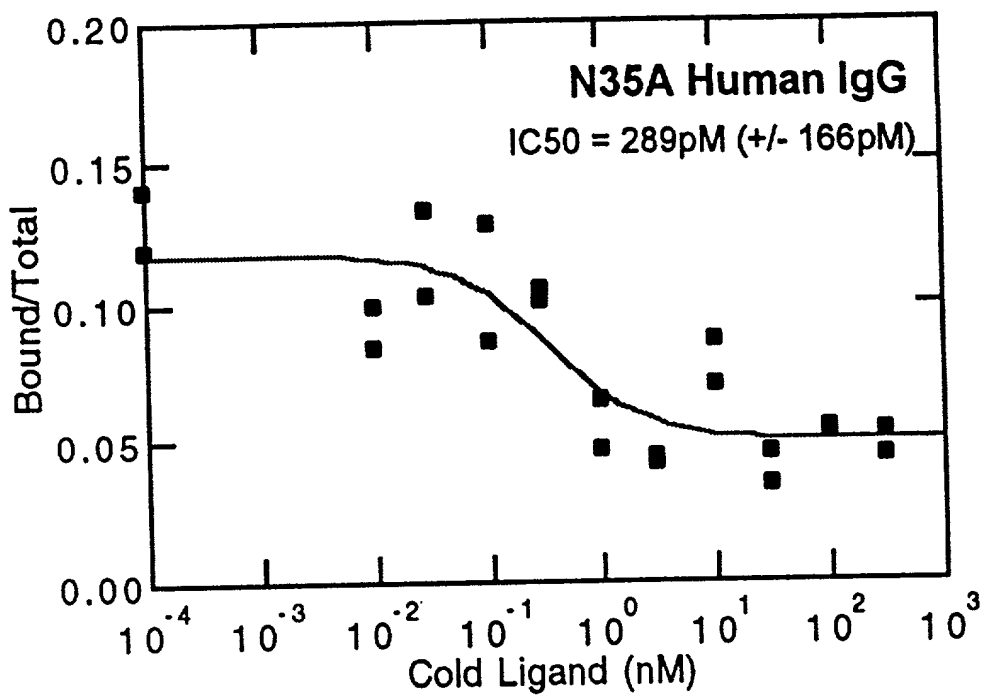
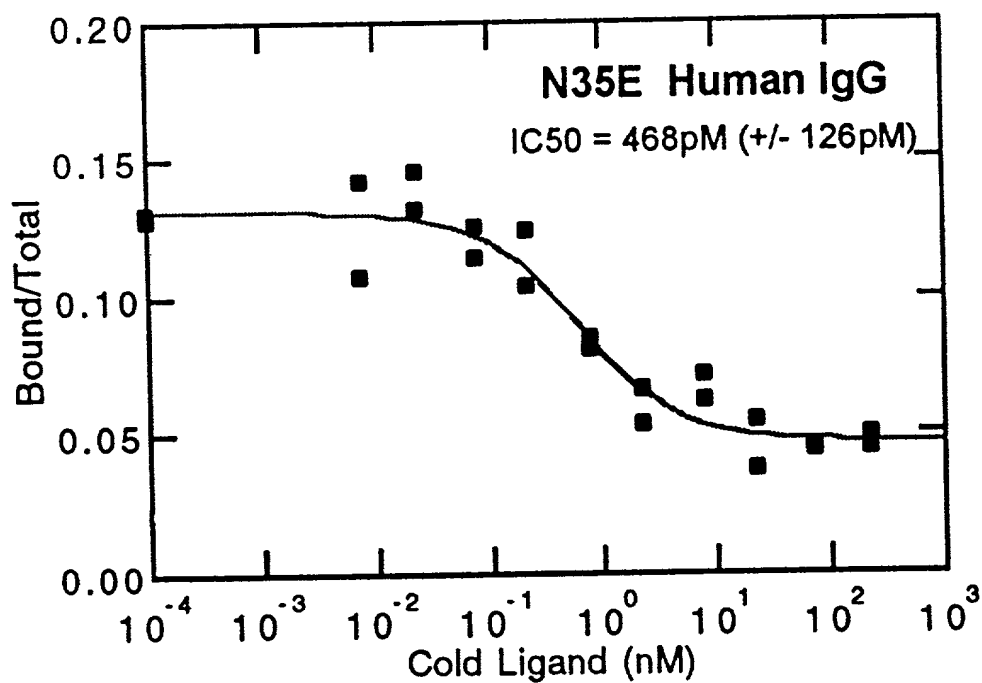


FIG. 49B



**FIG. 49C**



**FIG. 49D**

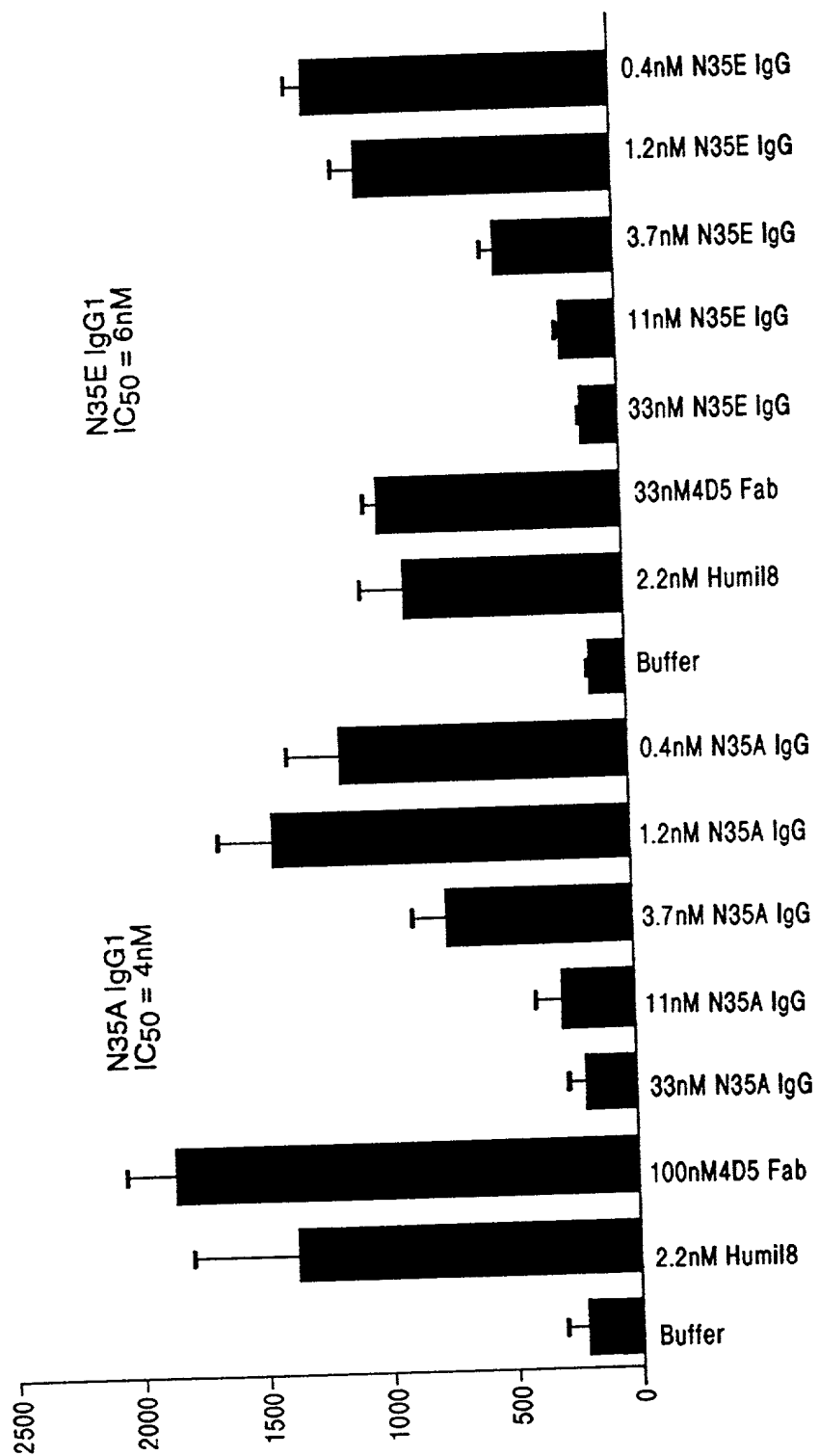


FIG. 50A

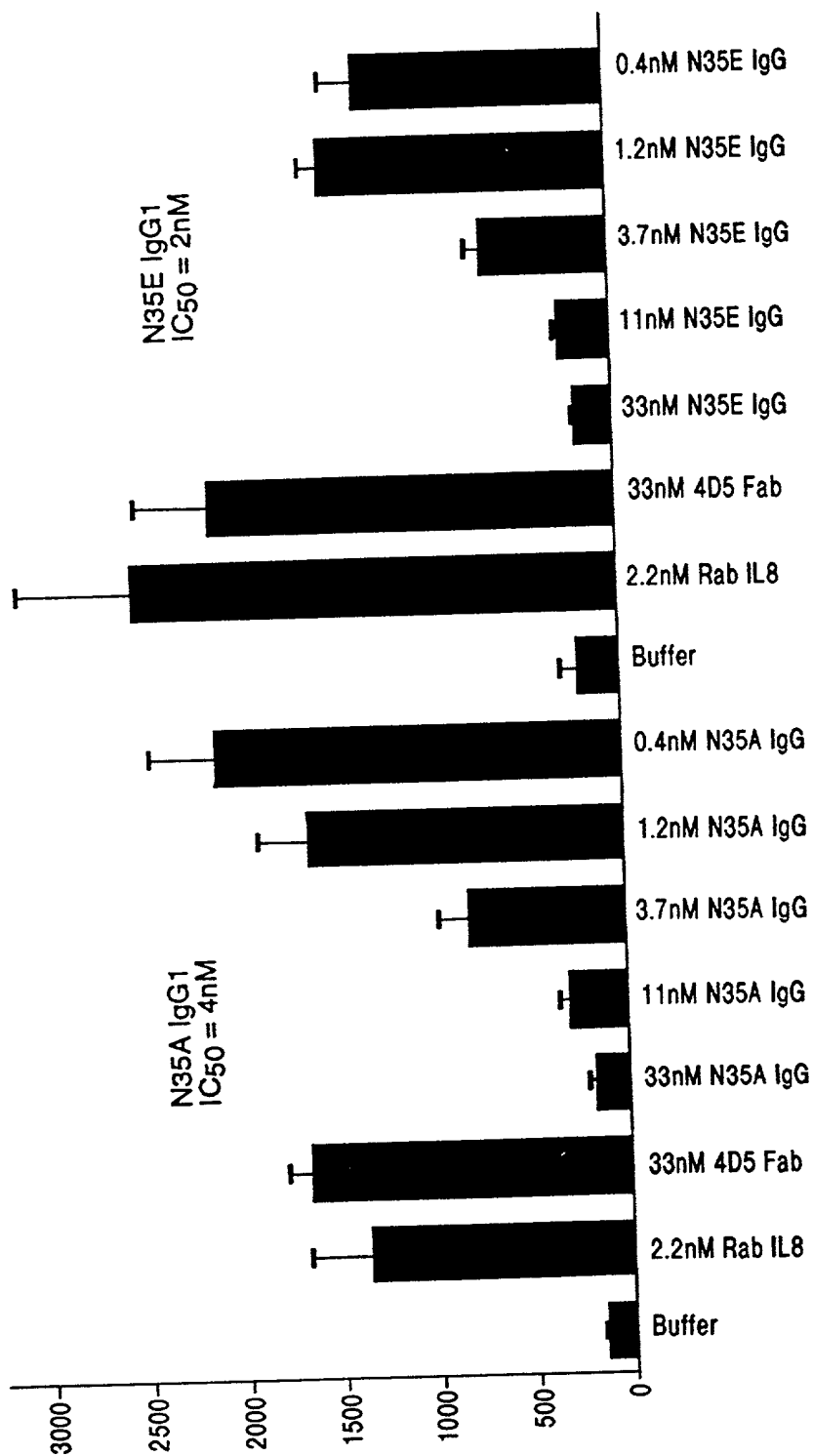
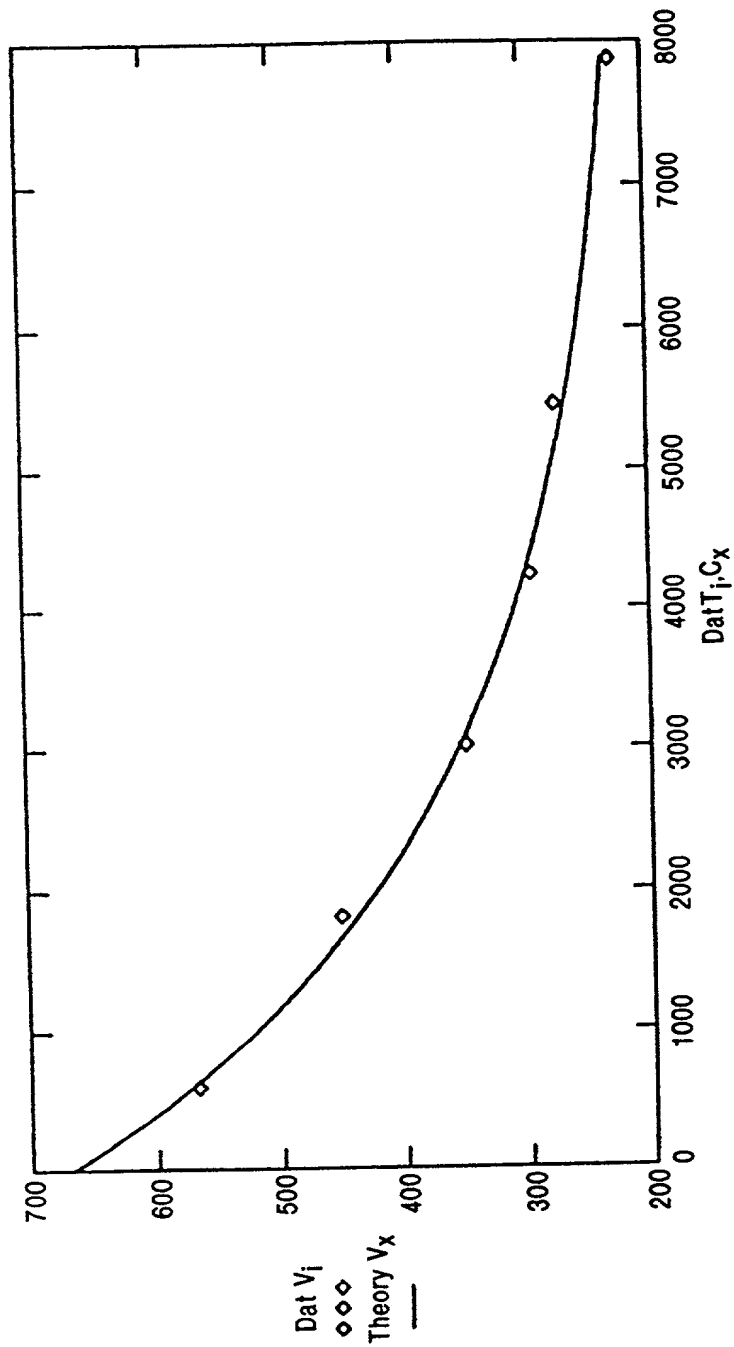


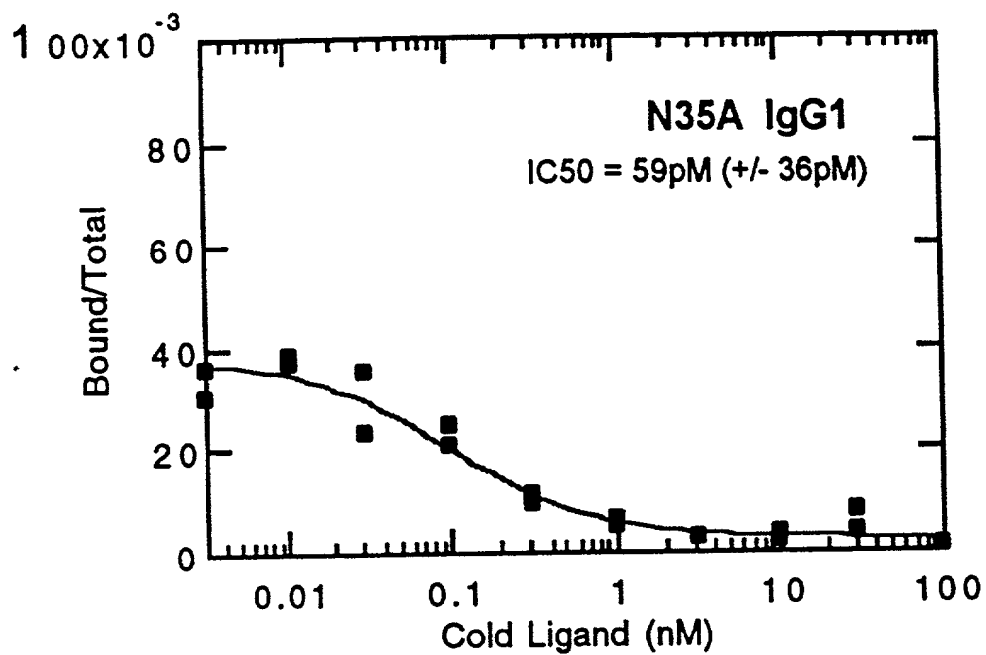
FIG. 50B



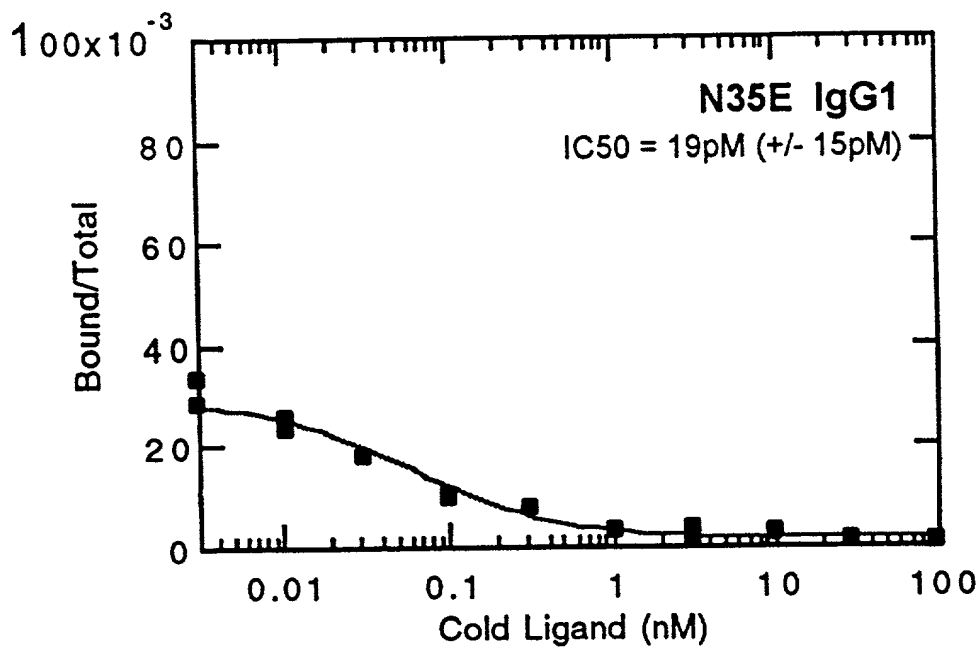
**Representative Conc versus Time Plot. Shown is the kinetic data for 6G4V11N35A.IgG1**

SAMPLE	ka	kd	Kd
Murine 6G4.2.5 IgG2a	8.3x10 <sup>5</sup>	2.9x10 <sup>-4</sup>	350pM
6G4V11N35A-IgG1	8.7x10 <sup>5</sup>	7.7x10 <sup>-5</sup>	88pM
6G4V11N35E-IgG1	3.0x10 <sup>6</sup>	1.4x10 <sup>-4</sup>	49pM

**FIG. 51**



**FIG. 52A**



**FIG. 52B**



781 AAAAGGGTAT CTAGAGGTTG AGGTGATTTT ATGAAAAAGA ATATCGCATT TCTTCTTGCA  
 TTTTCCCATA GATCTCCAAC TCCACTAAAA TACTTTTCT TATAGCGTAA AGAAGAACGT  
 -1 M K K N I A F L L A  
 841 TCTATGTTTCG TTTTCTCTAT TGCTACAAAC GCGTACGCTG AGGTTTCAGCT AGTGCAGTCT  
 AGATACAAGC AAAAAAGATA ACGATGTTTG CGCATGCGAC TCCAAGTCGA TCACGTCAGA  
 -11 S M F V F S I A T N A Y A E V Q L V Q S  
 901 GCGGTGGCC TGGTGCAGCC AGGGGGCTCA CTCCGTTTGT CCTGTGCAGC TTCTGGCTAC  
 CCGCCACCGG ACCACGTCGG TCCCCGAGT GAGGCAAACA GGACACGTCG AAGACCGATG  
 8 G G G L V Q P G G S L R L S C A A S G Y  
 961 TCCTTCTCGA GTCACTATAT GCACTGGGTC CGTCAGGCC CCGGTAAGGG CCTGGAATGG  
 AGGAAGAGCT CAGTGATATA CGTGACCCAG GCAGTCCGGG GCCCATTCCC GGACCTTACC  
 28 S F S S H Y M H W V R Q A P G K G L E W  
 1021 GTTGGATATA TTGATCCTTC CAATGGTGAA ACTACGTATA ATCAAAAGTT CAAGGGCCGT  
 CAACCTATAT AACTAGGAAG GTTACCACTT TGATGCATAT TAGTTTTCAA GTTCCCGGCA  
 48 V G Y I D P S N G E T T Y N O K F K G R  
 1081 TTCACTTTAT CTCGCGACAA CTCCAAAAAC ACAGCATACC TGCAGATGAA CAGCCTGCGT  
 AAGTGAAATA GAGCGCTGTT GAGGTTTTTG TGTCGTATGG ACGTCTACTT GTCGGACGCA  
 68 F T L S R D N S K N T A Y L Q M N S L R  
 1141 GCTGAGGACA CTGCCGTCTA TTA CTGTGCA AGAGGGGATT ATCGCTACAA TGGTGACTGG  
 CGACTCCTGT GACGGCAGAT AATGACACGT TCTCCCCTAA TAGCGATGTT ACCACTGACC  
 88 A E D T A V Y Y C A R G D Y R Y N G D W  
 1201 TTCTTCGACG TCTGGGGTCA AGGAACCCTG GTCACCGTCT CCTCGGCCTC CACCAAGGGC  
 AAGAAGCTGC AGACCCAGT TCCTTGGGAC CAGTGGCAGA GGAGCCGAG GTGGTTCCCG  
 108 F F D V W G Q G T L V T V S S A S T K G  
 1261 CCATCGGTCT TCCCCCTGGC ACCCTCCTCC AAGAGCACCT CTGGGGGCAC AGCGGCCCTG  
 GG TAGCCAGA AGGGGGACCG TGGGAGGAGG TTCTCGTGGA GACCCCGTG TCGCCGGGAC  
 128 P S V F P L A P S S K S T S G G T A A L  
 1321 GGCTGCCTGG TCAAGGACTA CTTCCCCGAA CCGGTGACGG TGTCGTGGAA CTCAGGCGCC  
 CCGACGGACC AGTTCTTGAT GAAGGGGCTT GGCCACTGCC ACAGCACCTT GAGTCCGCGG  
 148 G C L V K D Y F P E P V T V S W N S G A  
 1381 CTGACCAGCG GCGTGCACAC CTTCCCGGCT GTCCTACAGT CCTCAGGACT CTACTCCCTC  
 GACTGGTCGC CGCACGTGTG GAAGGGCCGA CAGGATGTCA GGAGTCCTGA GATGAGGGAG  
 168 L T S G V H T F P A V L Q S S G L Y S L  
 1441 AGCAGCGTGG TGACCGTGCC CTCCAGCAGC TTGGGCACCC AGACCTACAT CTGCAACGTG  
 TCGTCGCACC ACTGGCACGG GAGGTCGTG AACCCTGGG TCTGGATGTA GACGTTGCAC  
 188 S S V V T V P S S S L G T Q T Y I C N V  
 1501 AATCACAAGC CCAGCAACAC CAAGGTGCAC AAGAAAGTTG AGCCCAAATC TTGTGACAAA  
 TTAGTGTTTCG GGTGCTGTG GTTCCAGCTG TTCTTTCAAC TCGGGTTTAG AACACTGTTT  
 208 N H K P S N T K V D K K V E P K S C D K  
 1561 ACTCACACAT GCGCGCGTGA  
 TGAGTGTGTA CGGGCGGCACT  
 228 T H T C P P O

FIG. 53

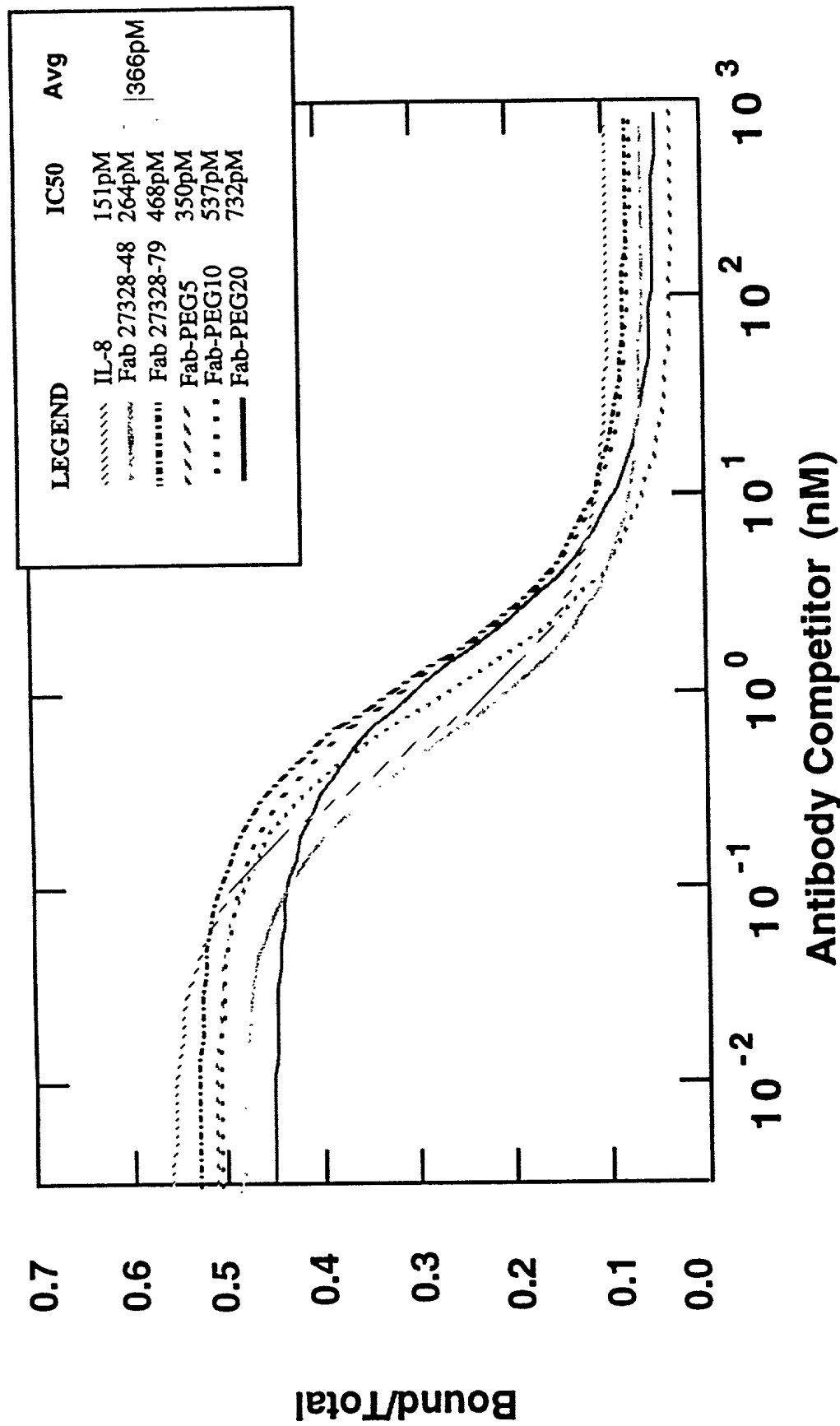


FIG. 54A

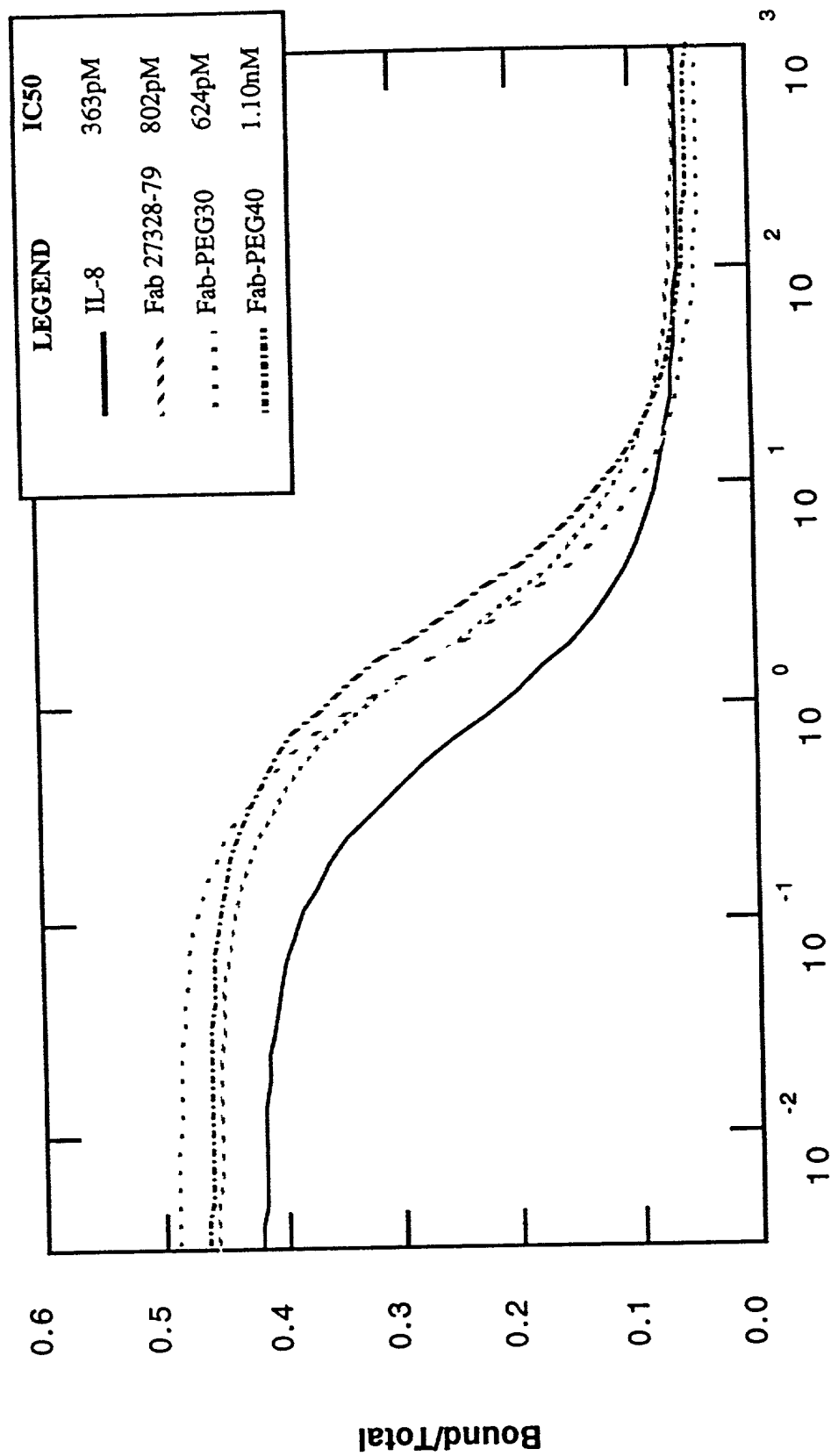
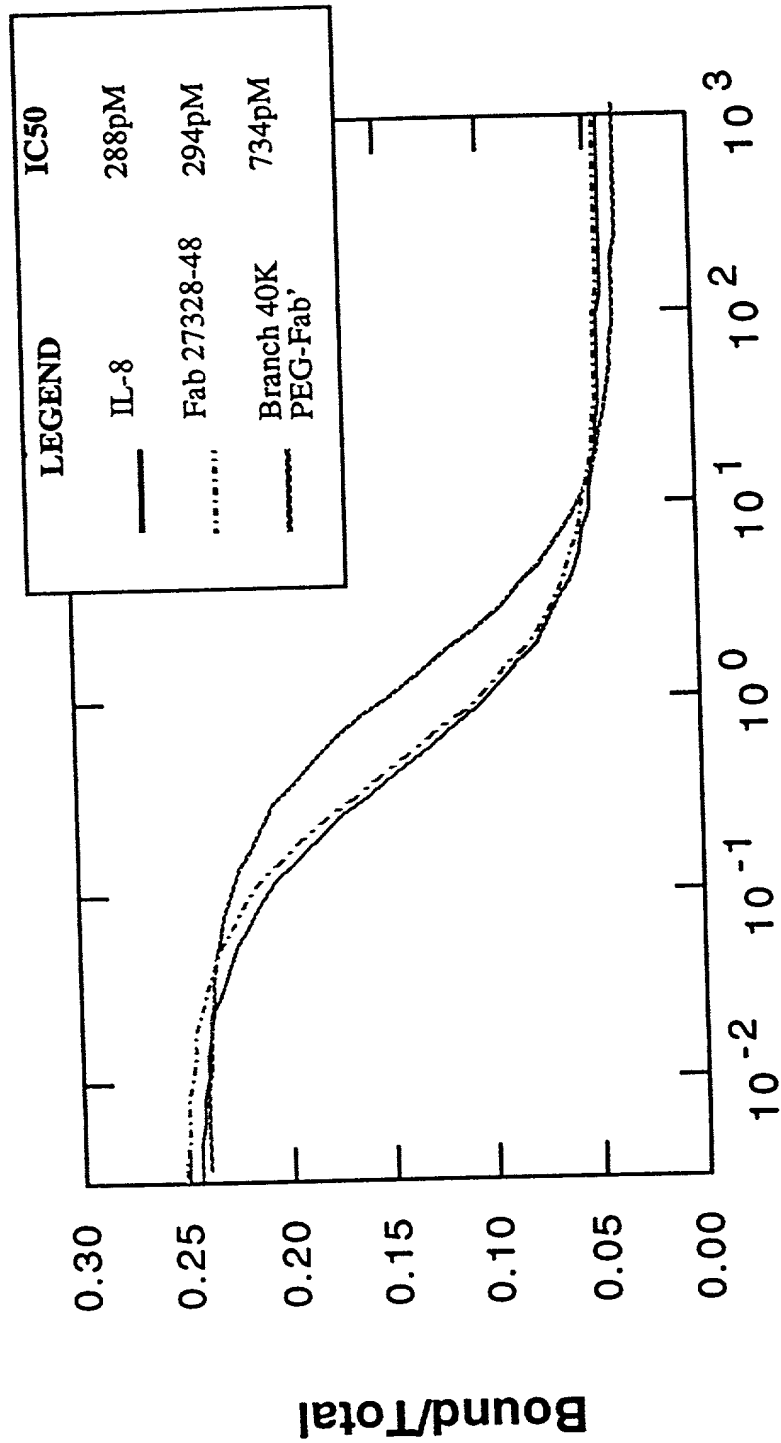


FIG. 54B



**FIG. 54C**

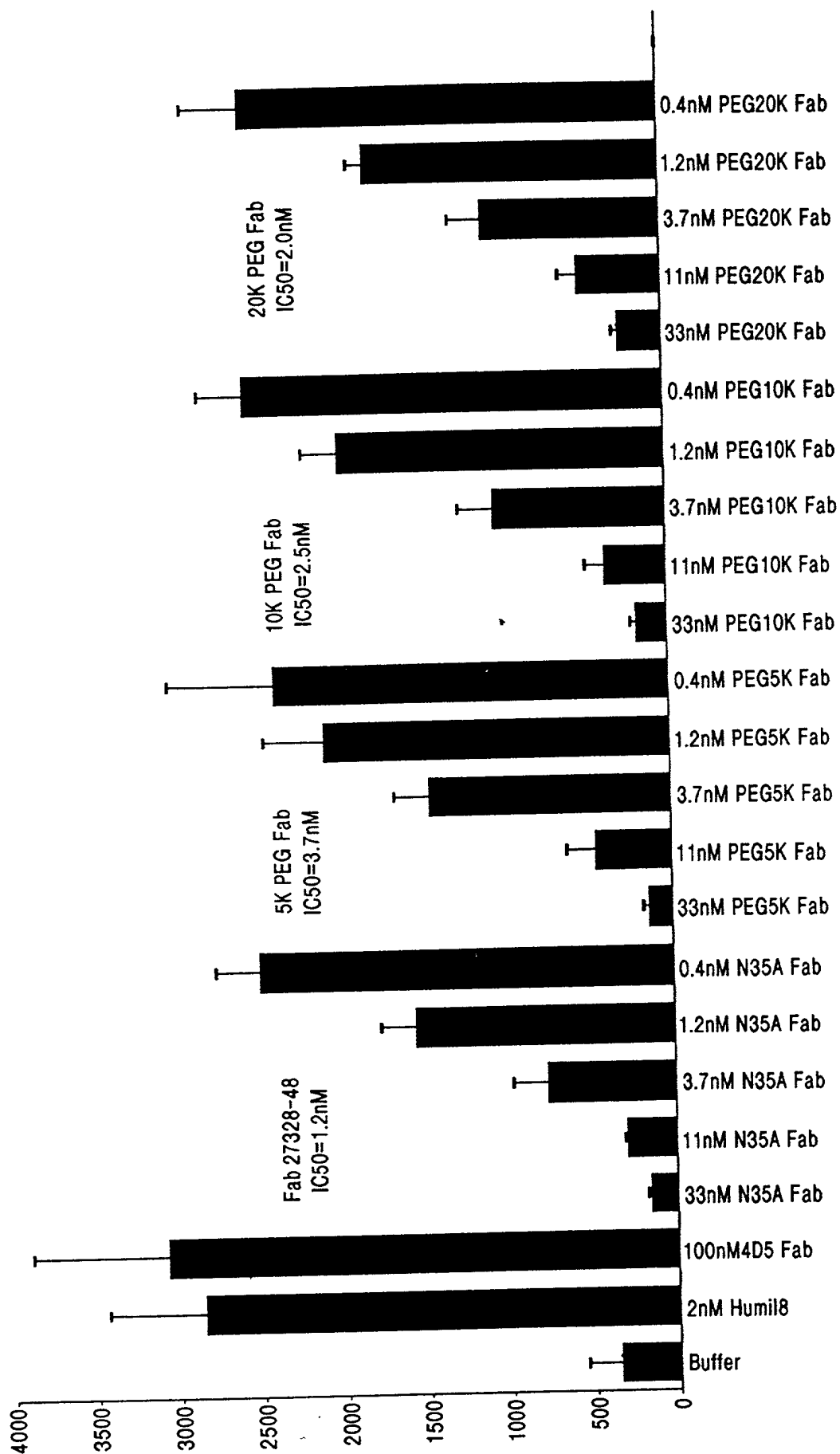


FIG. 55A

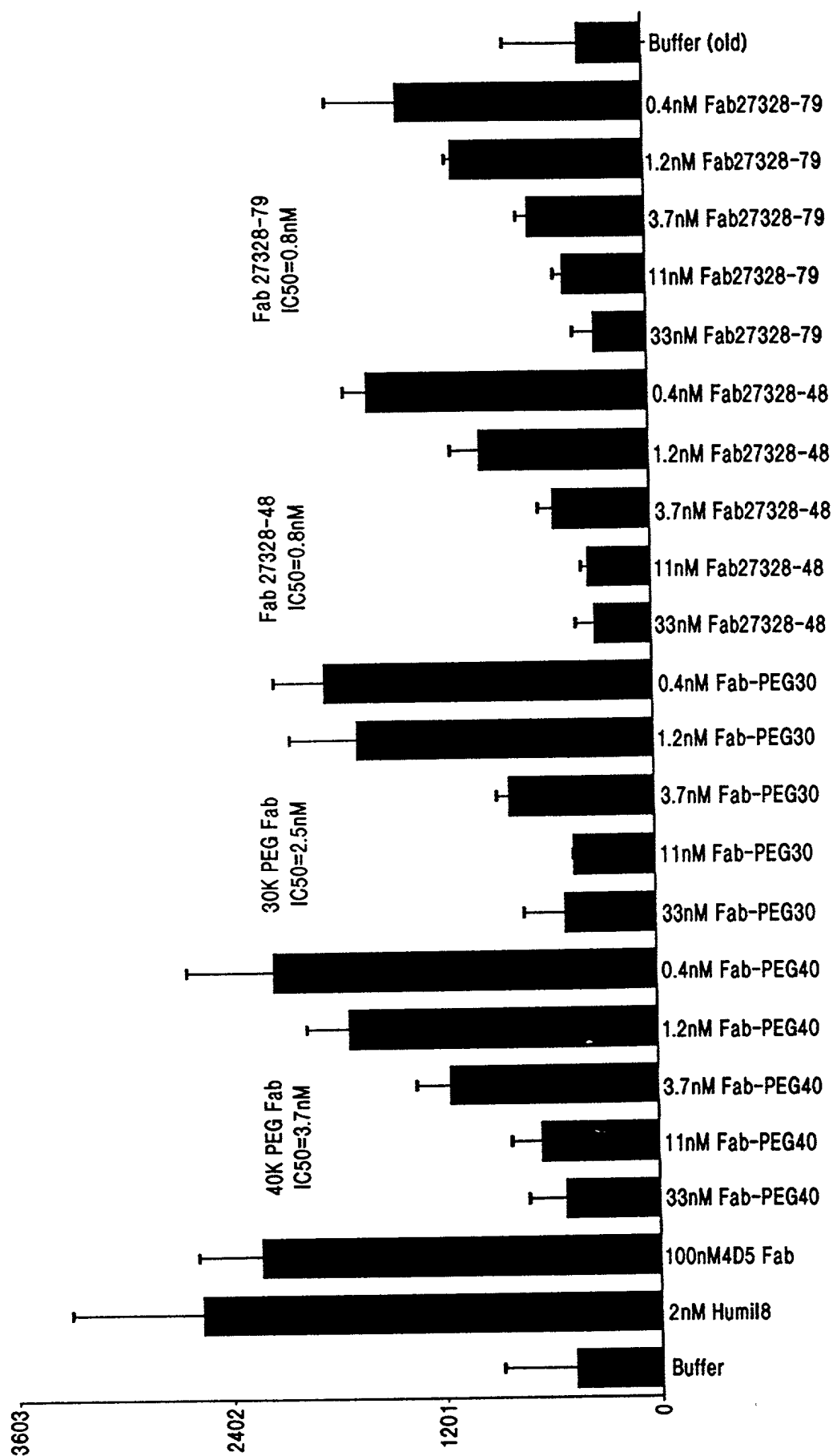


FIG. 55B

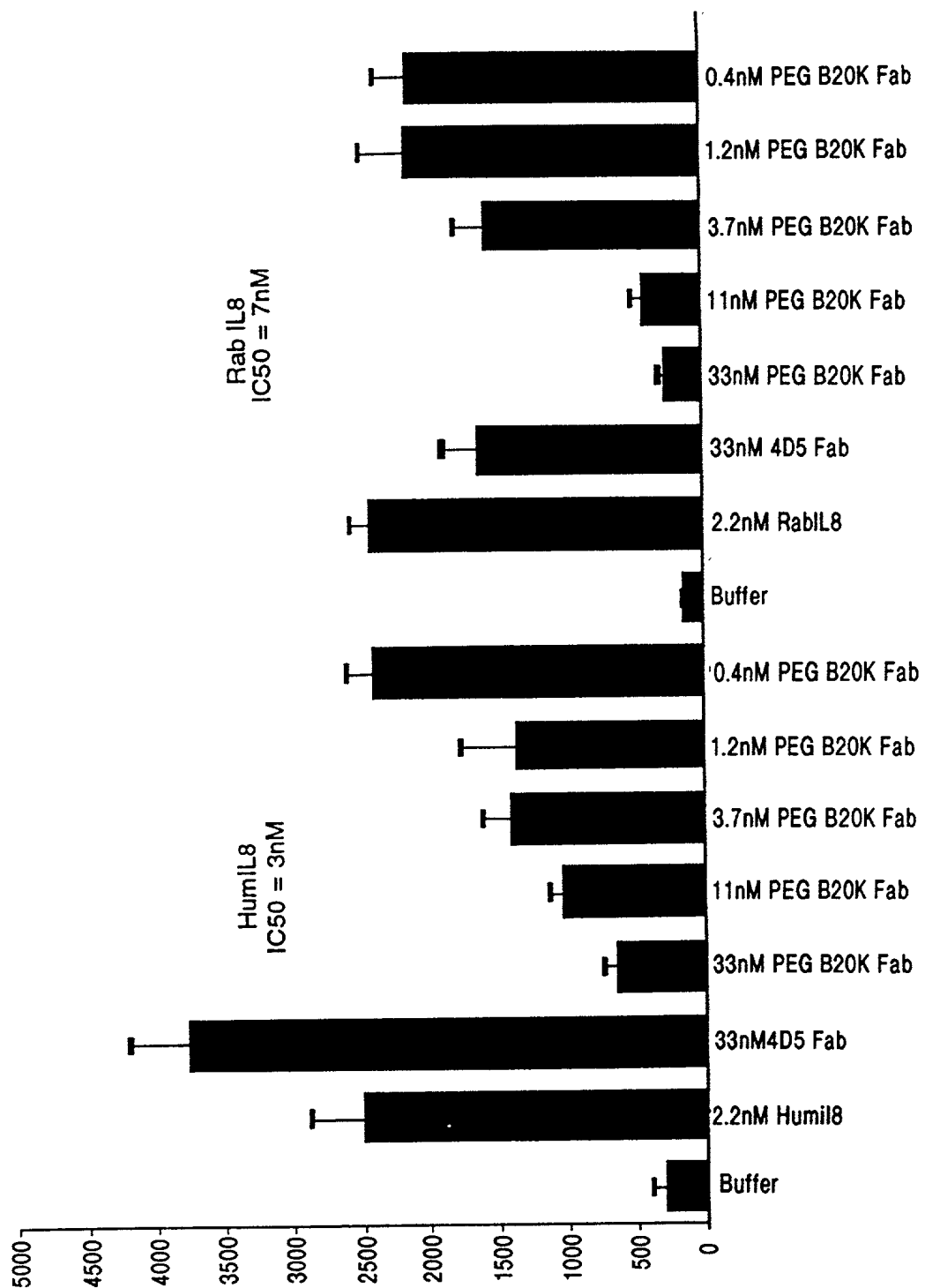
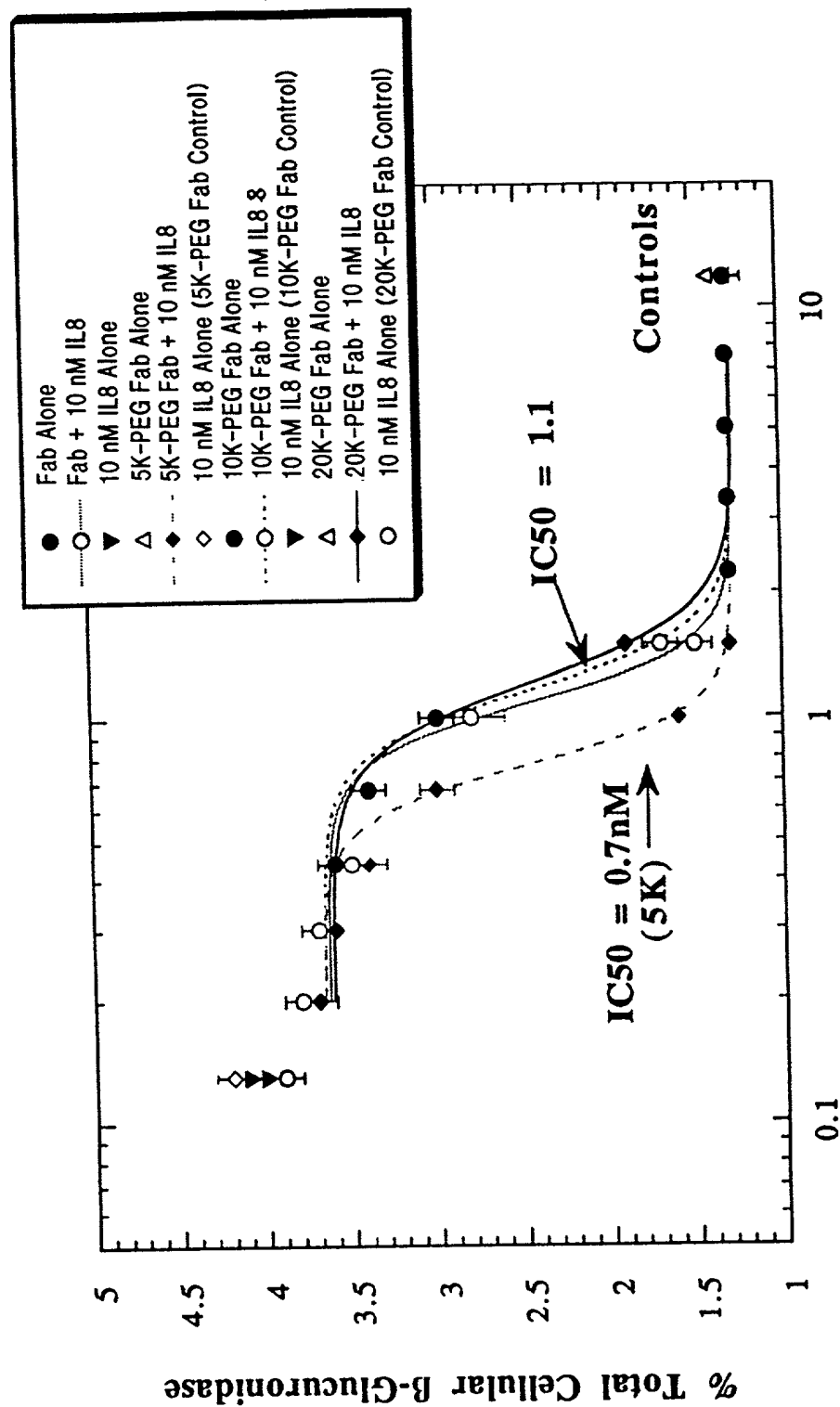


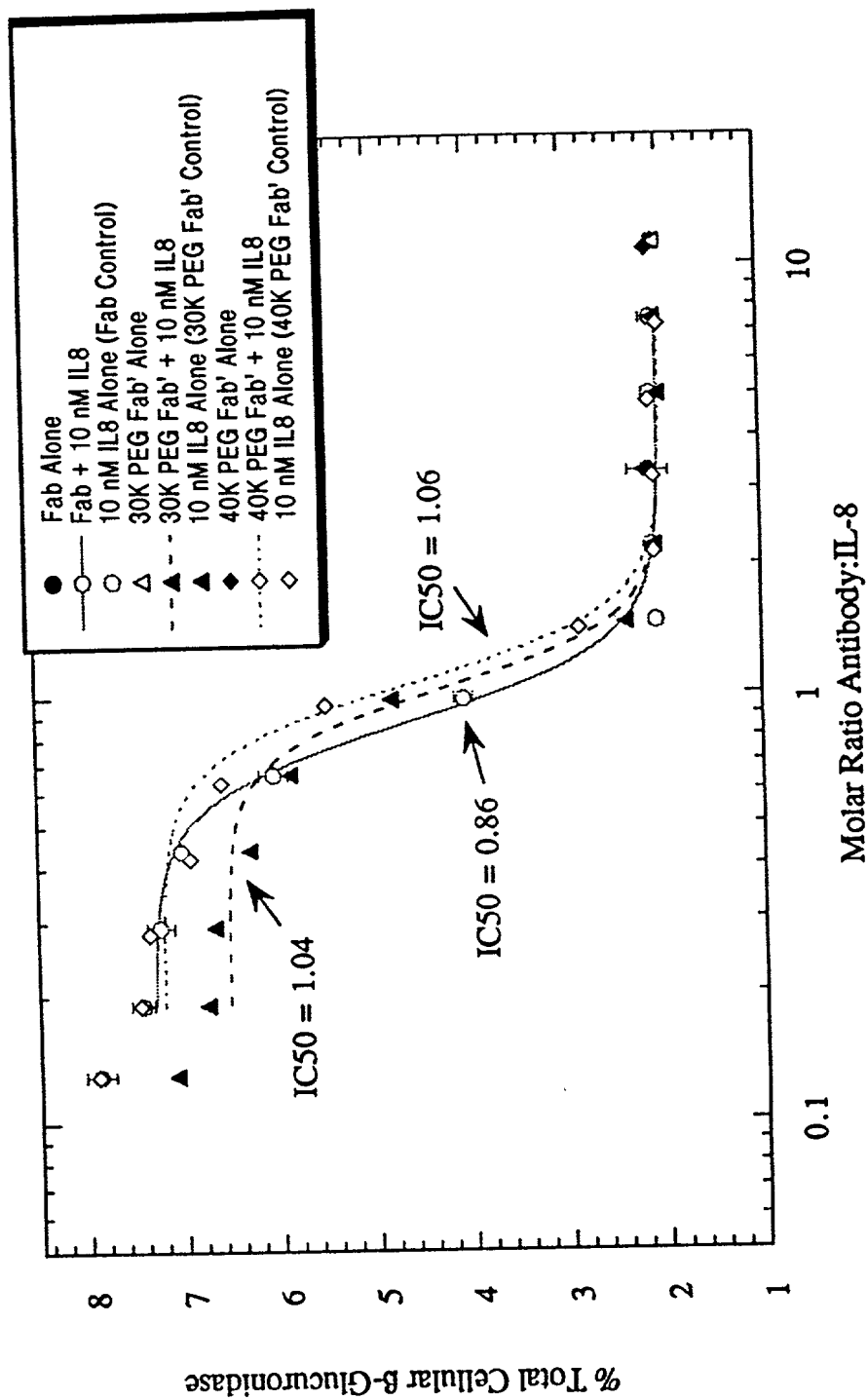
FIG. 55C



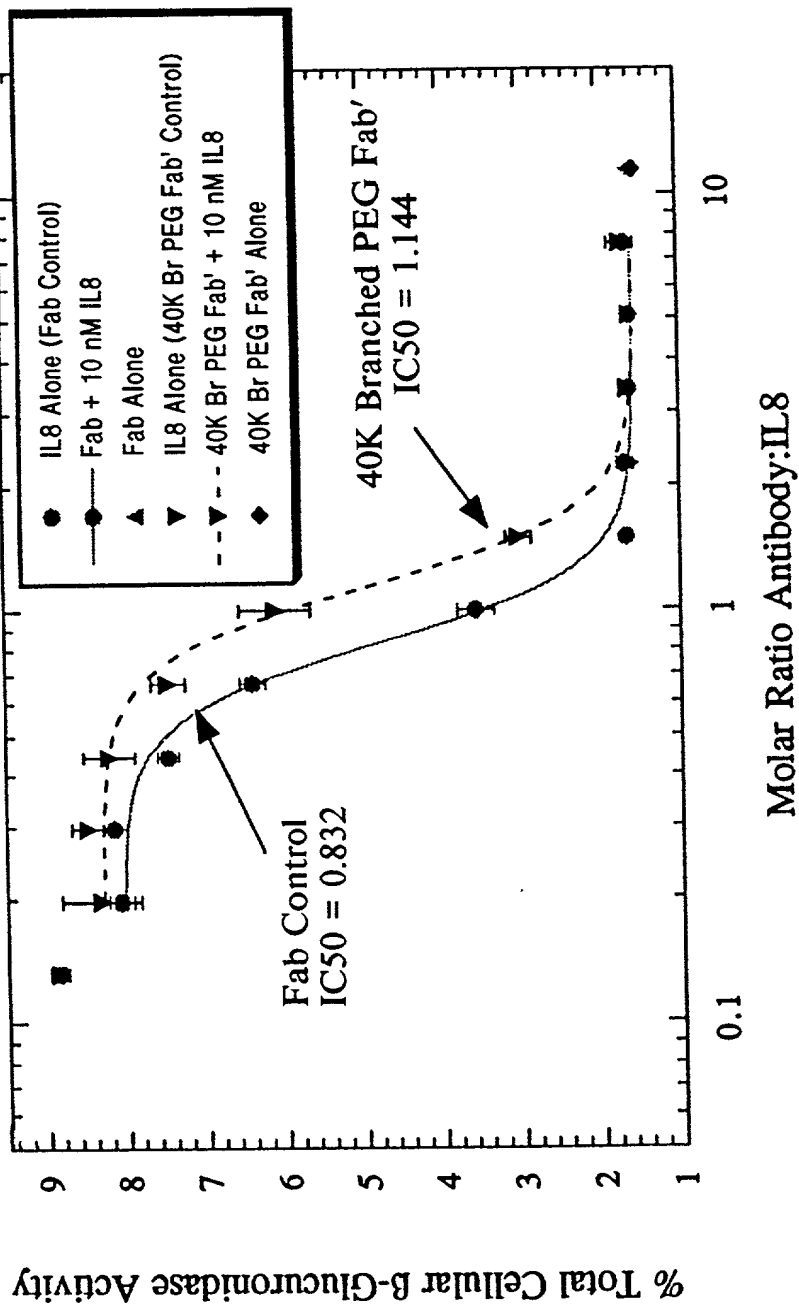
**Molar Ratio Antibody:IL-8**

**FIG. 56A**

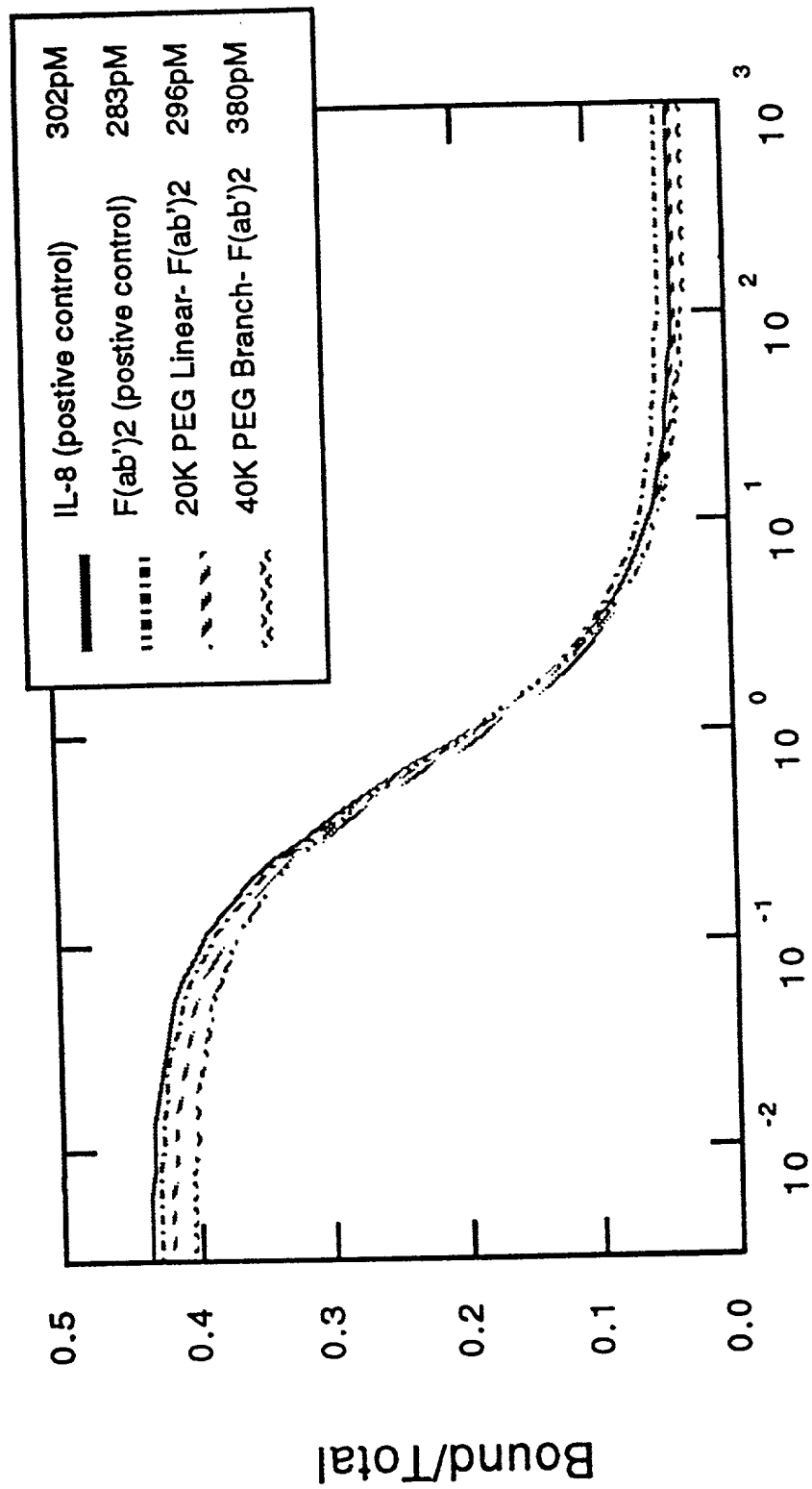




**FIG. 56B**

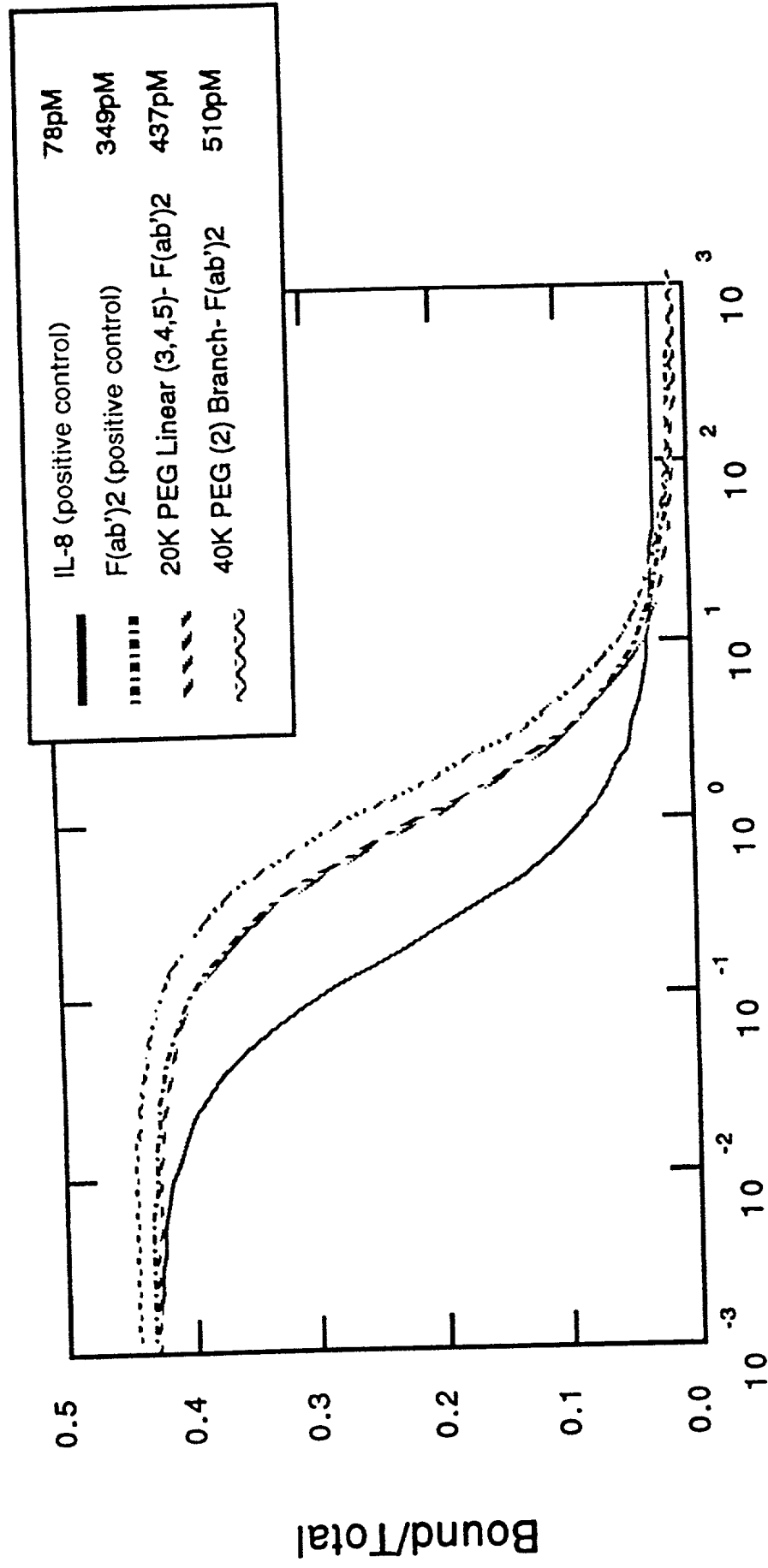


**FIG. 56C**



Pegylated F(ab')<sub>2</sub> (nM)

FIG. 57A



Pegylated F(ab')<sub>2</sub> (nM)

FIG. 57B

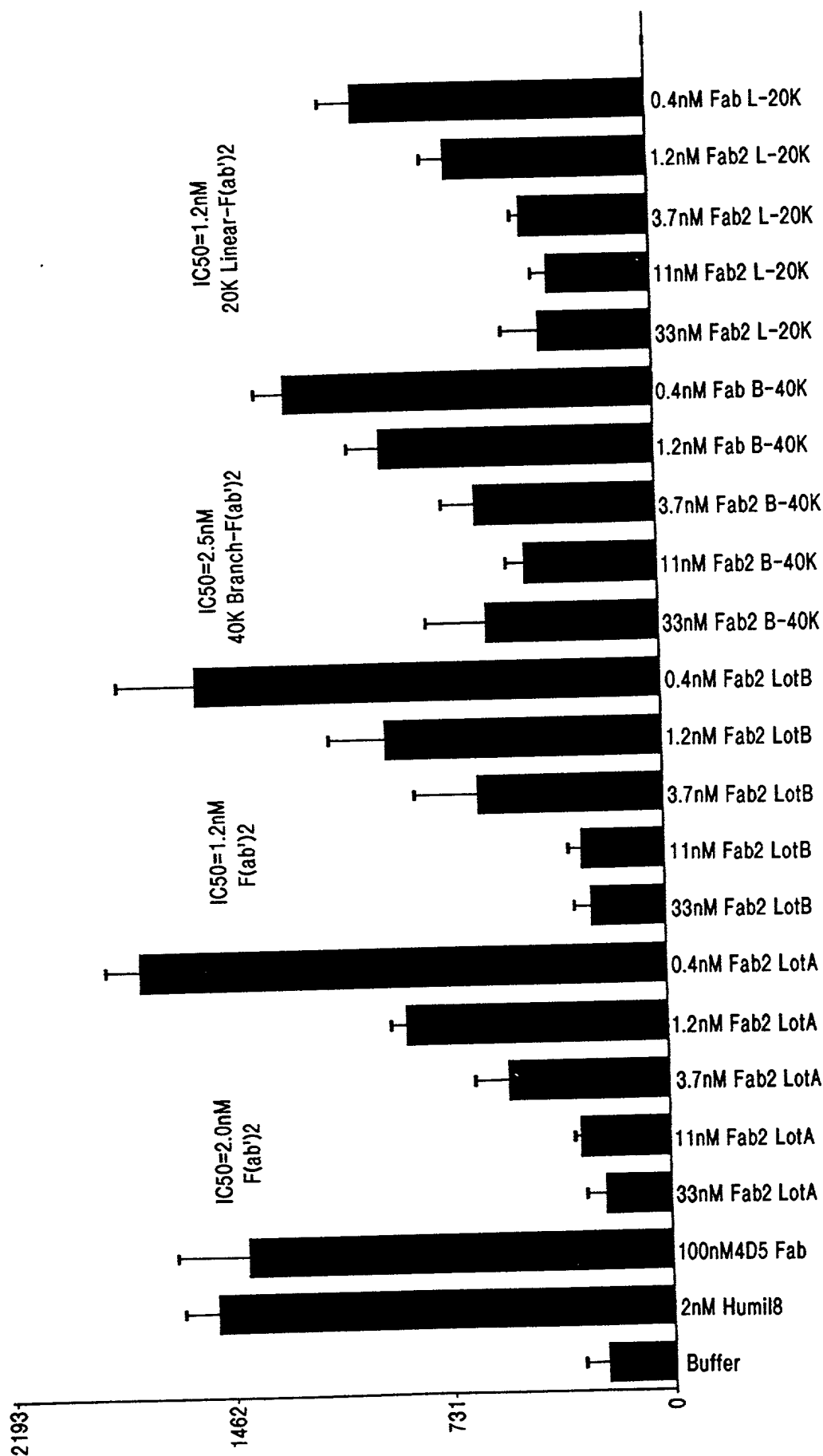


FIG. 58A

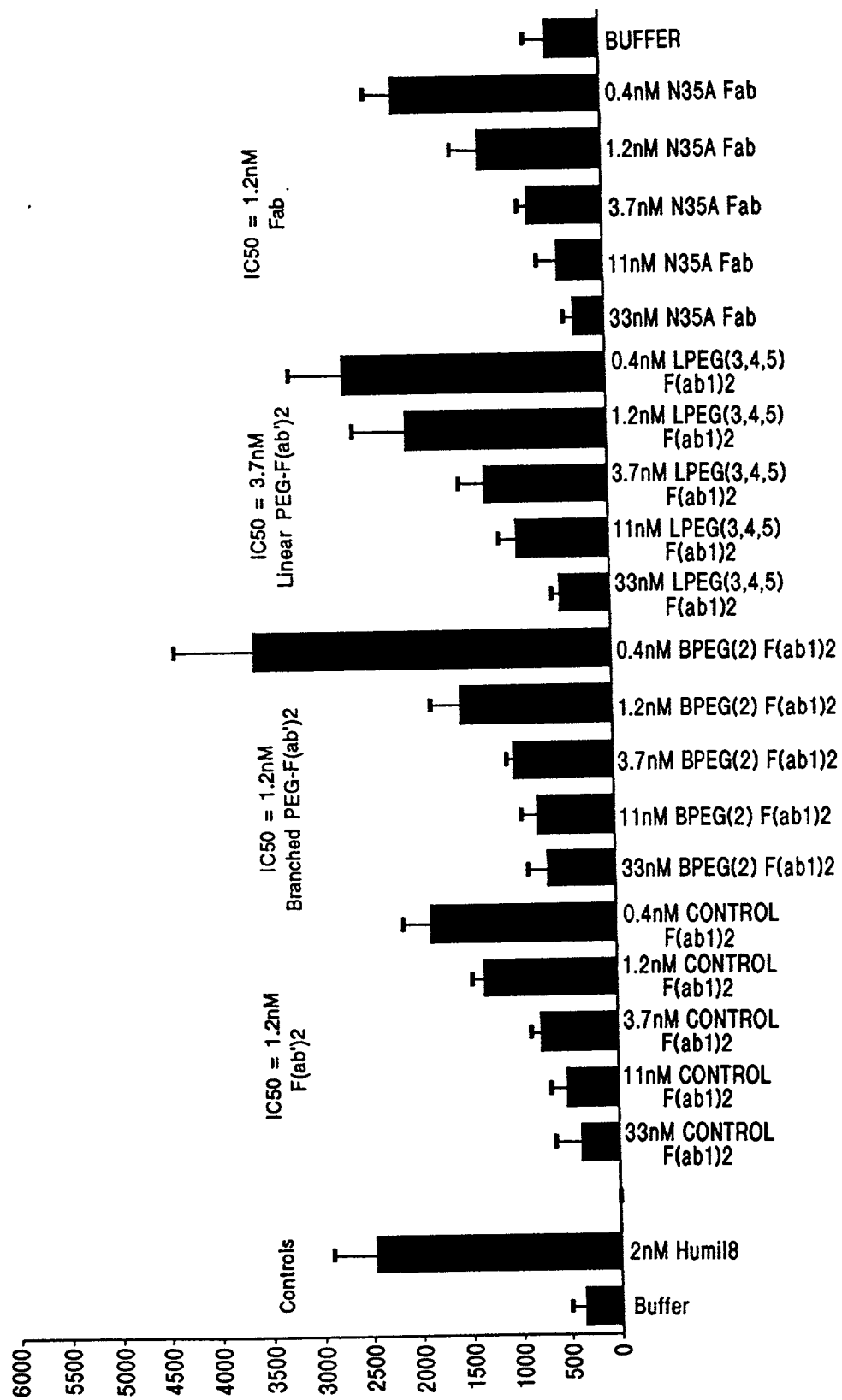


FIG. 58B

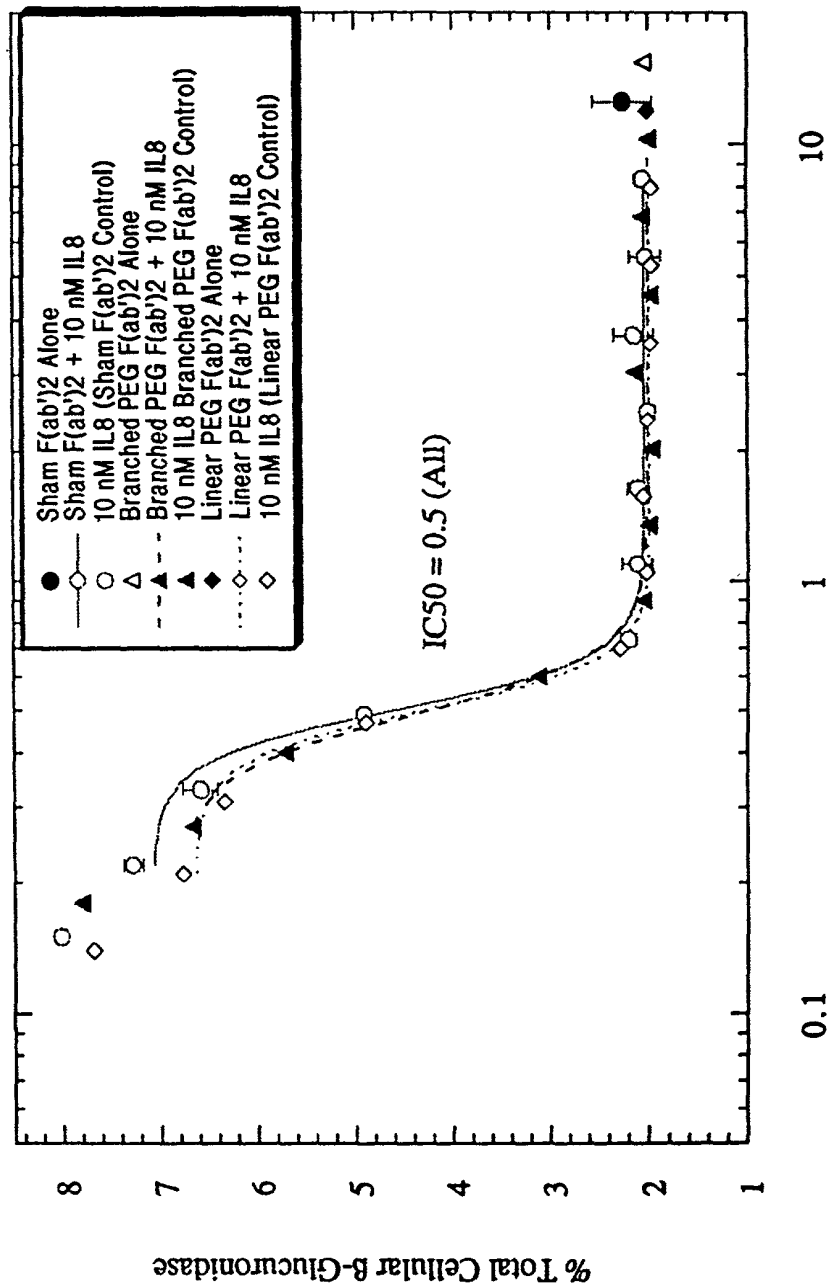
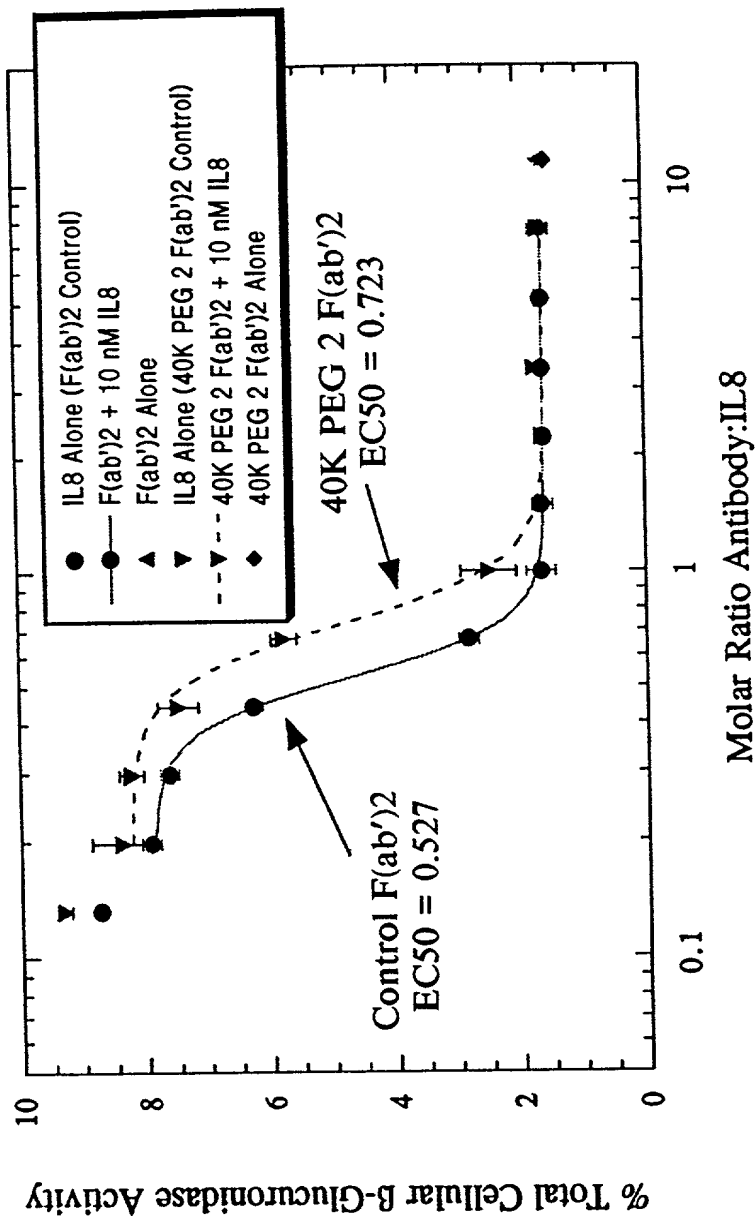
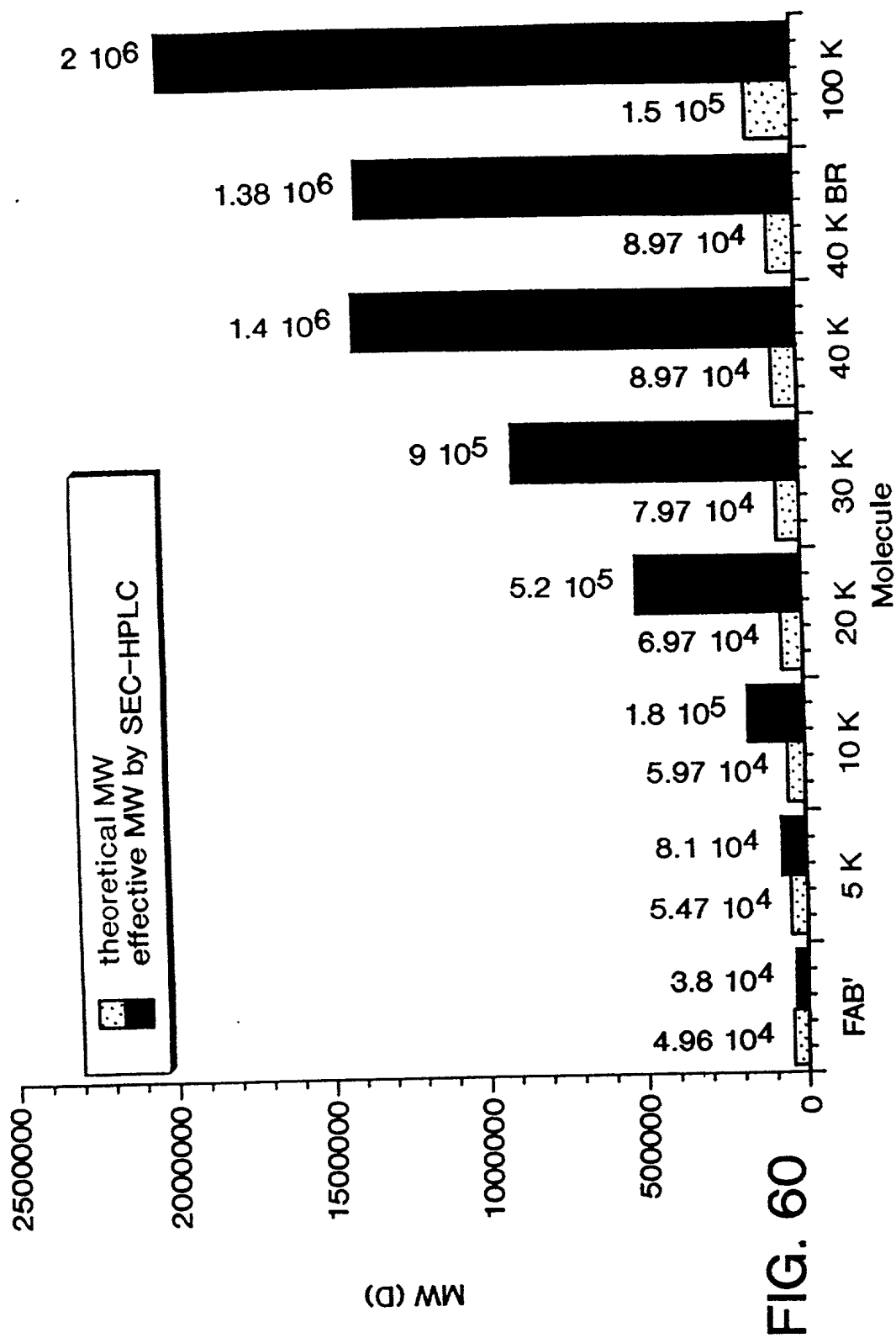


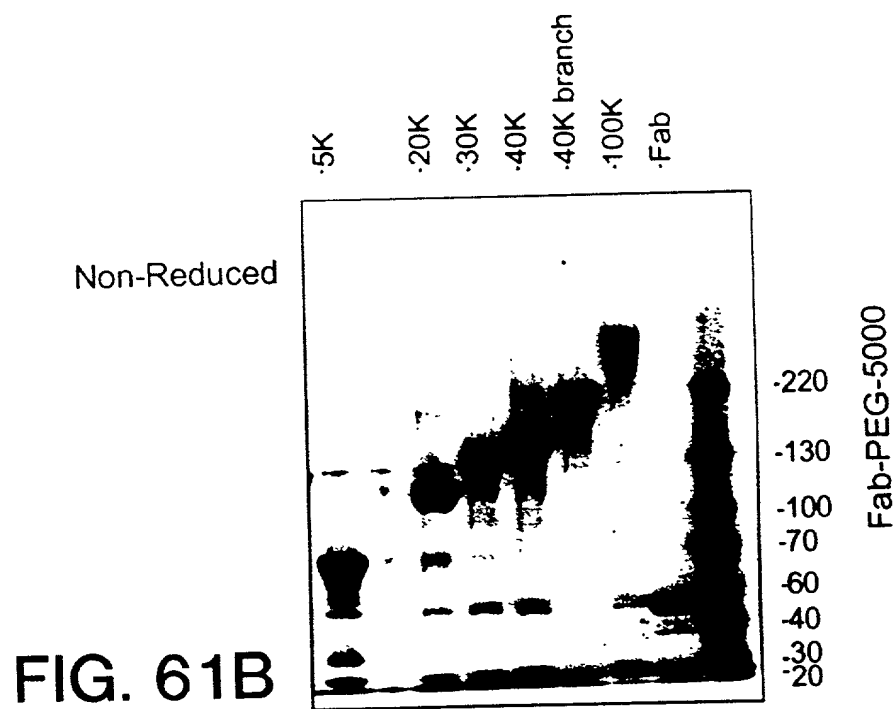
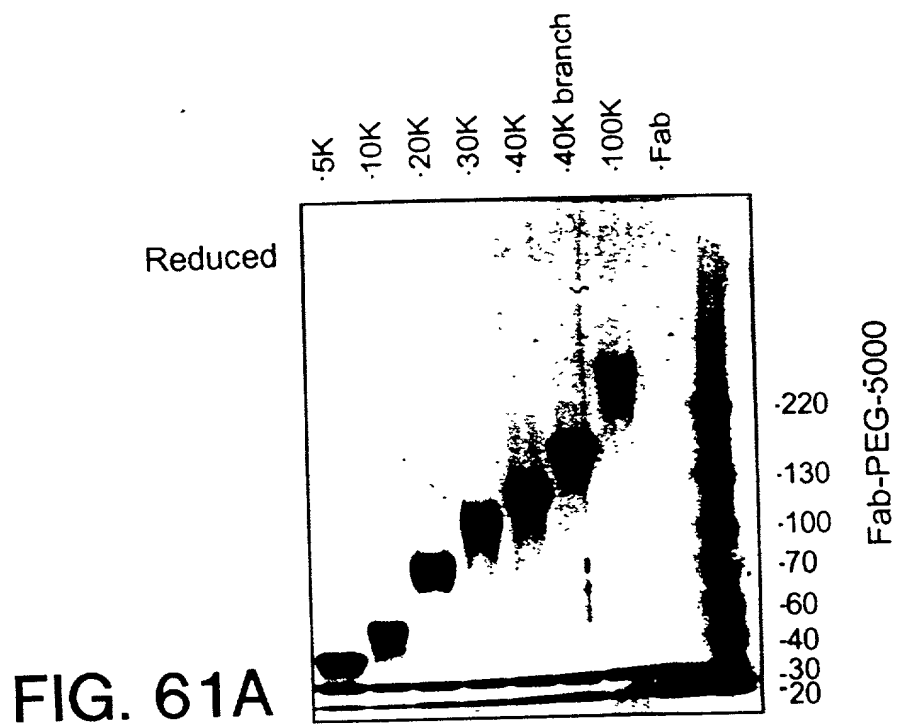
FIG. 59A



**FIG. 59B**







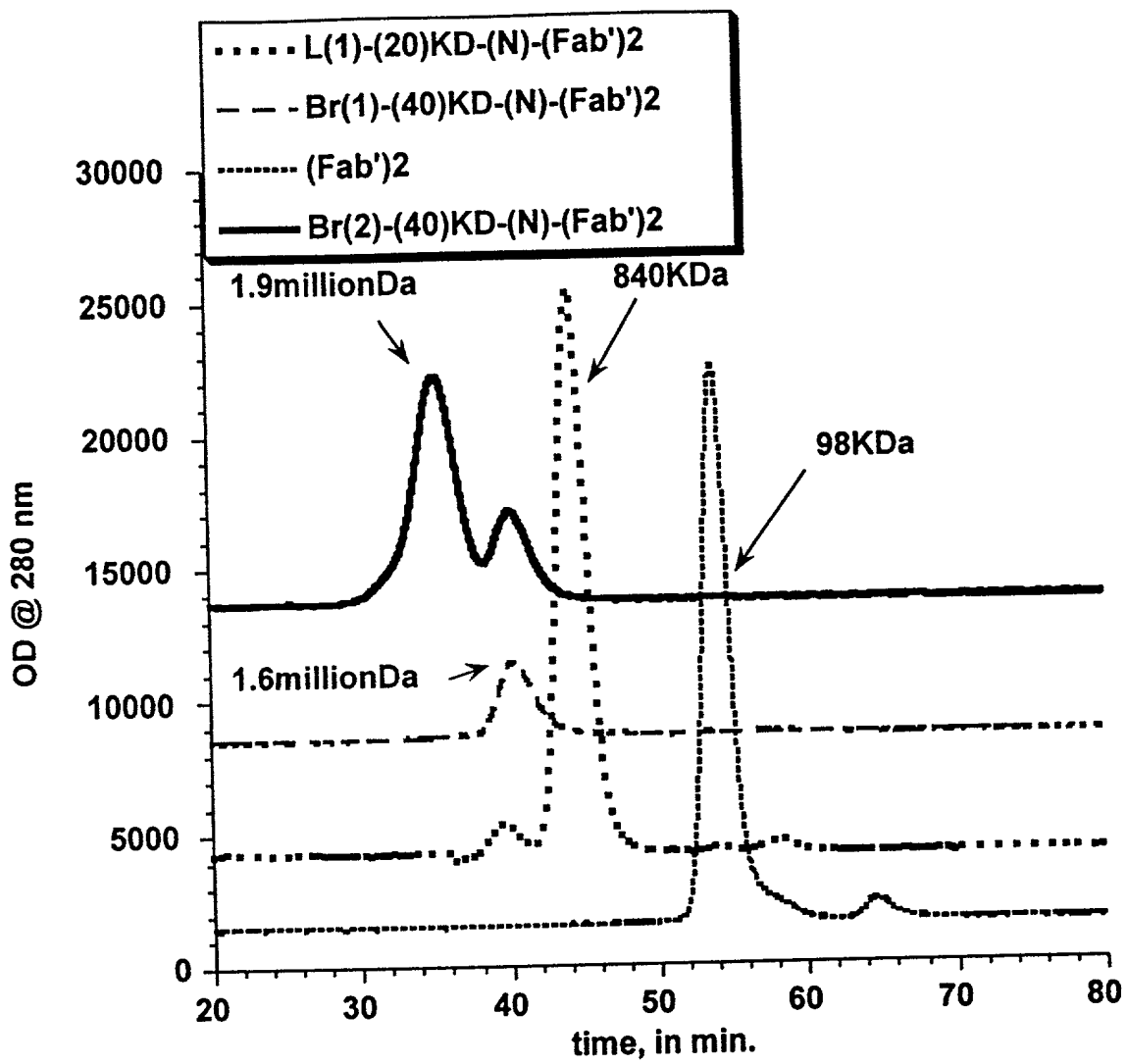


FIG. 62

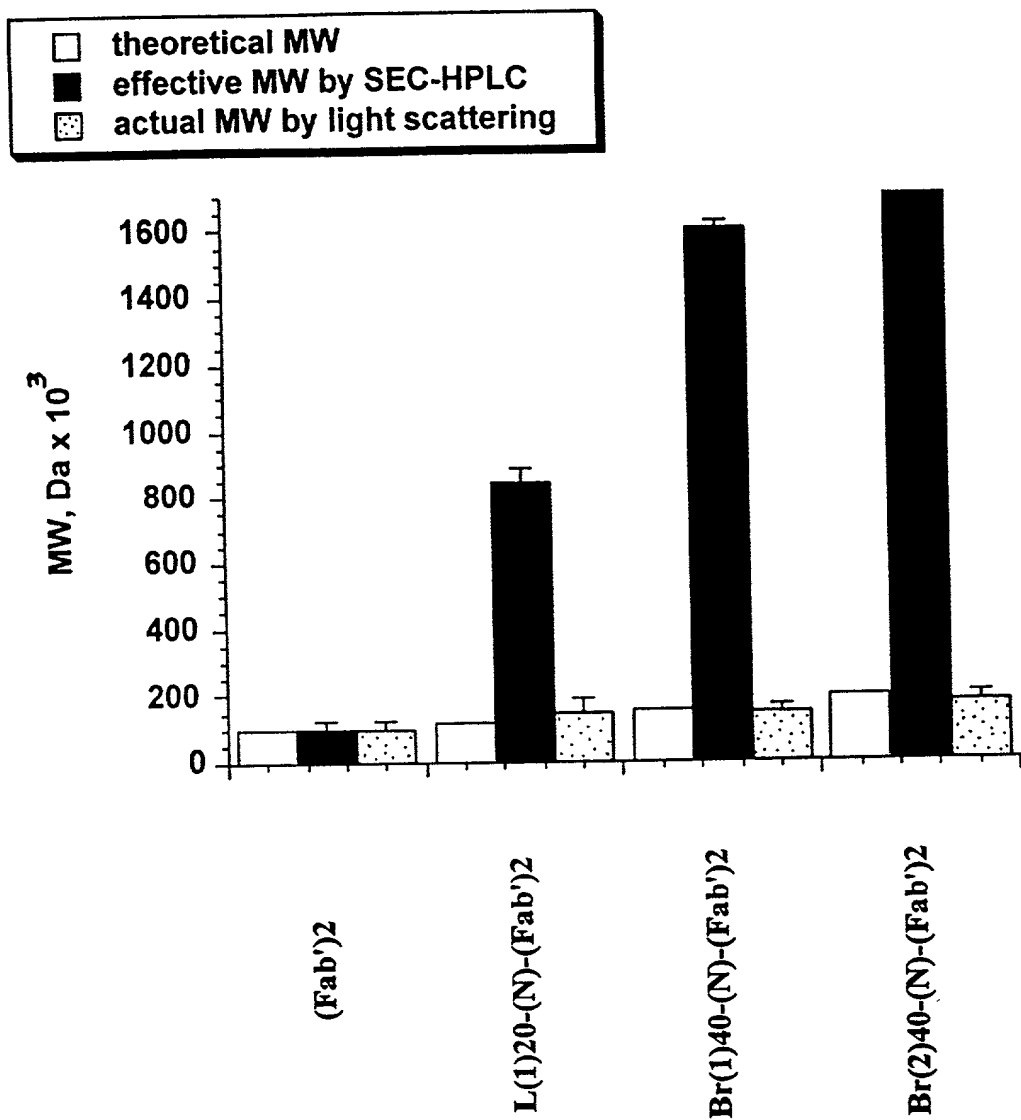


FIG. 63

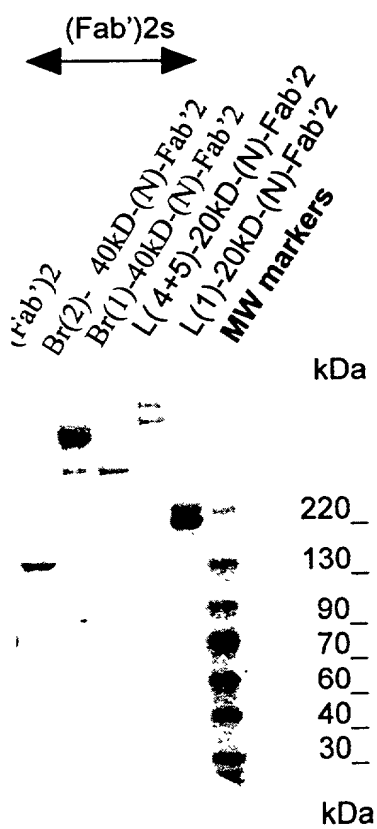
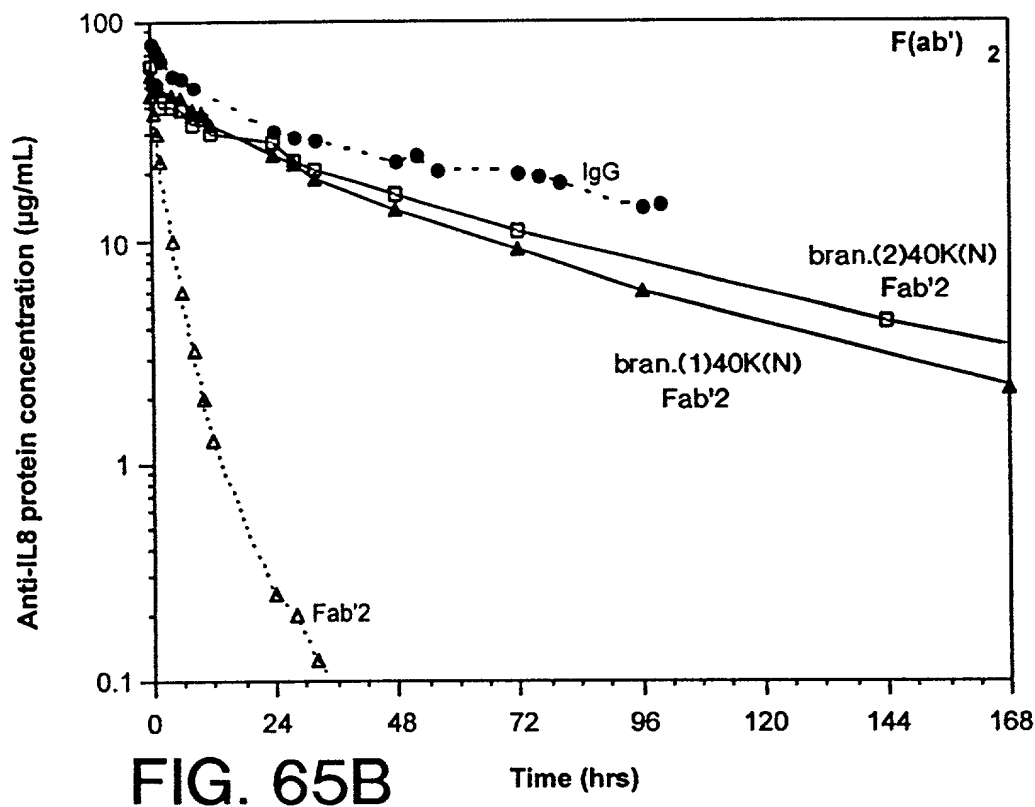
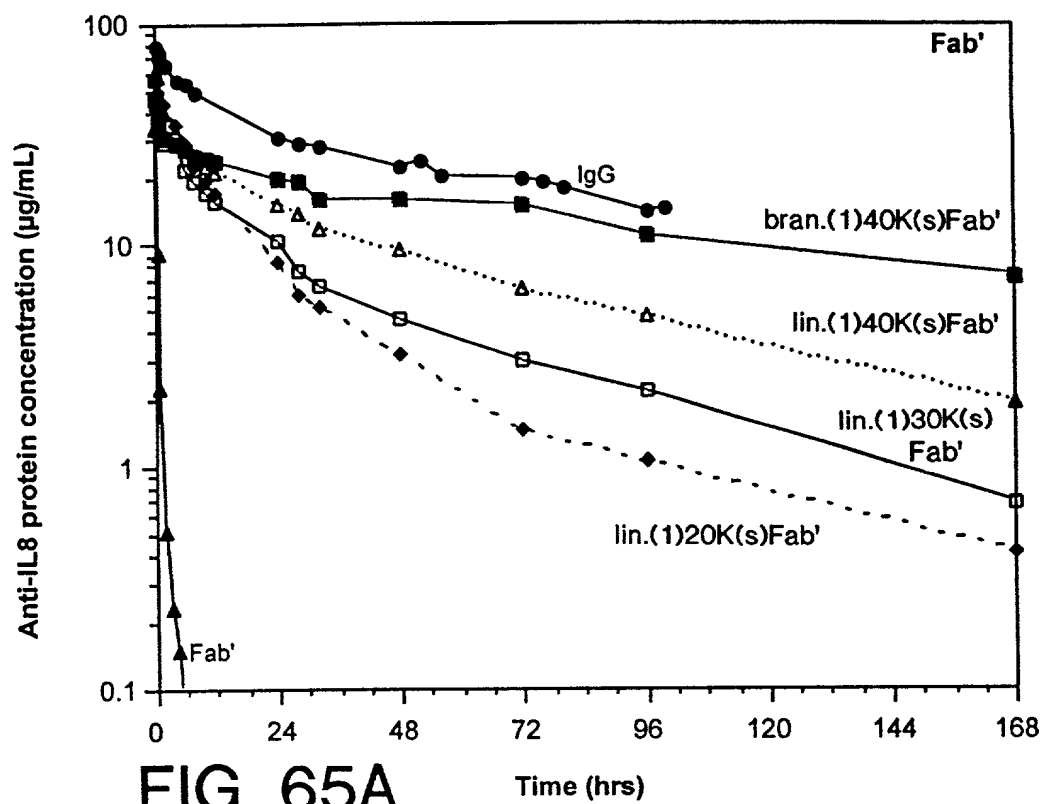


FIG. 64



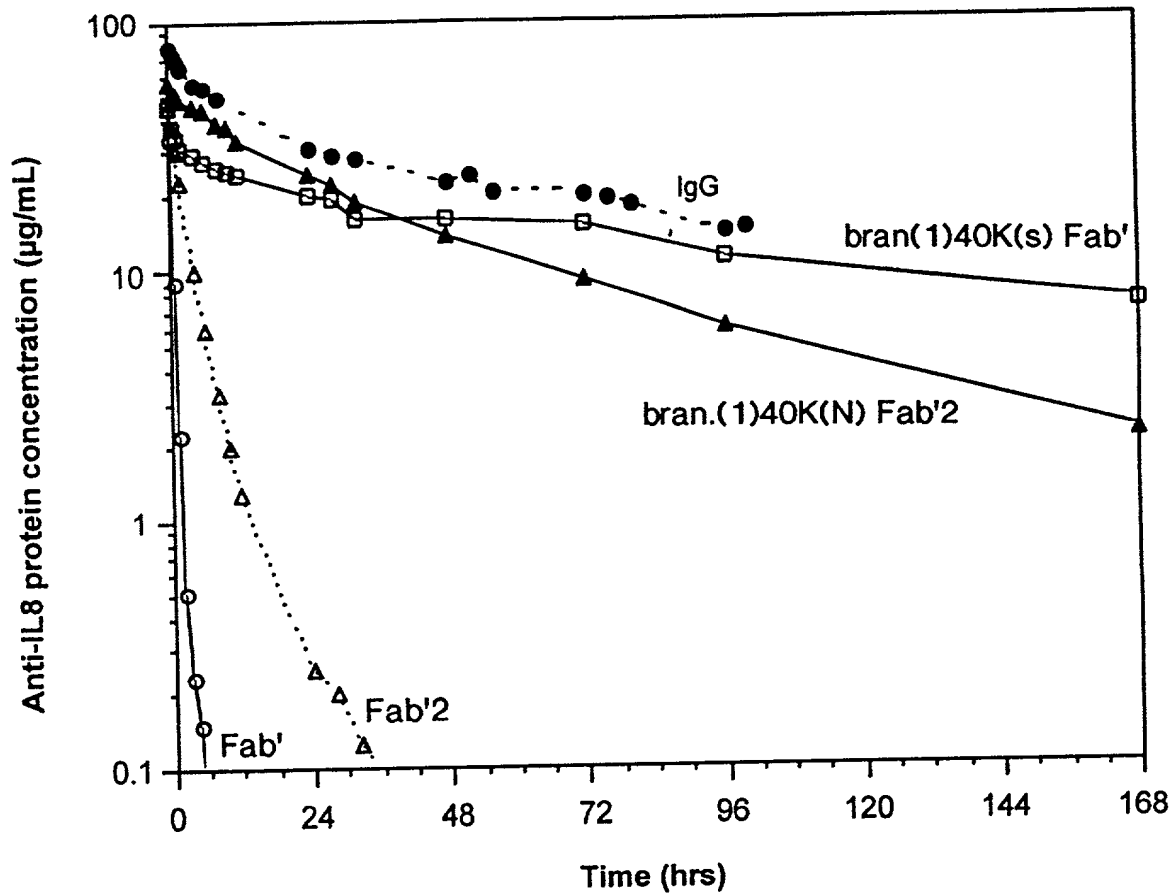


FIG. 66

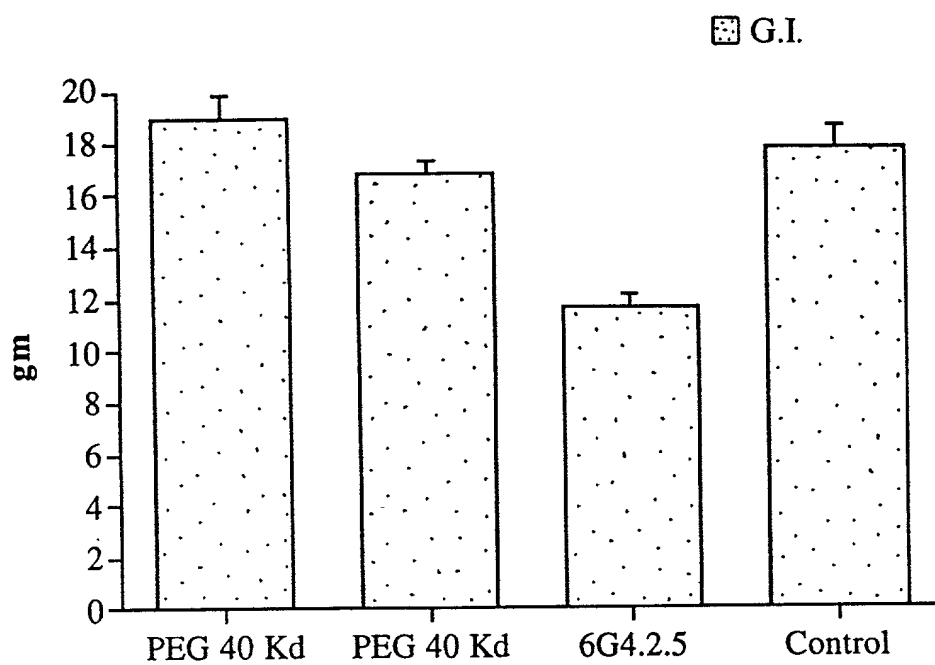


FIG. 67

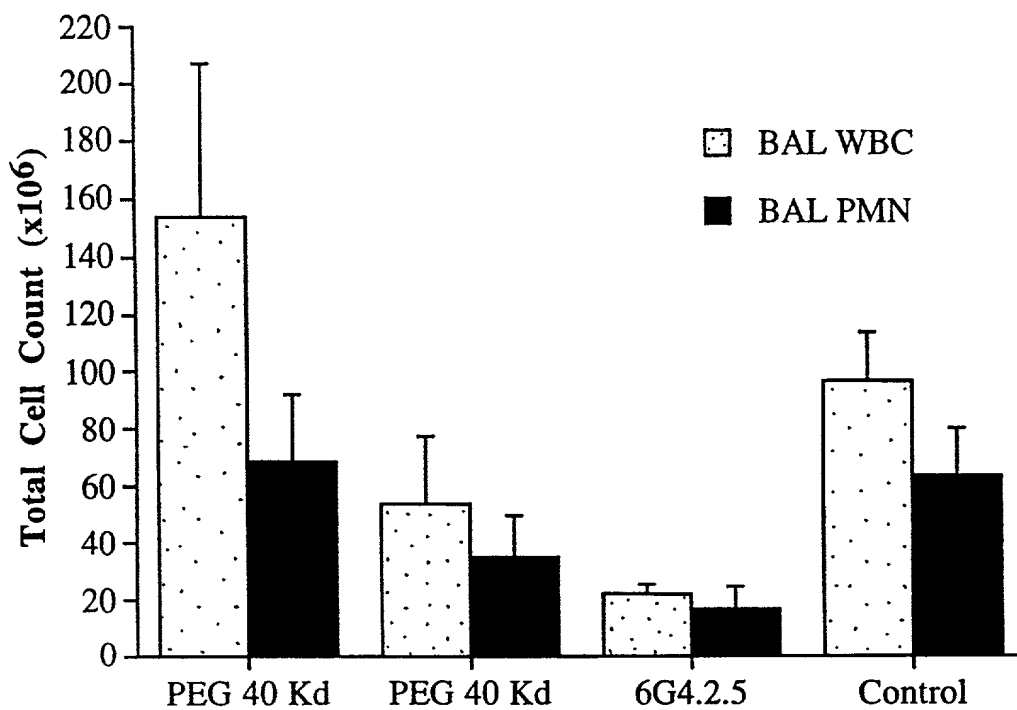


FIG. 68



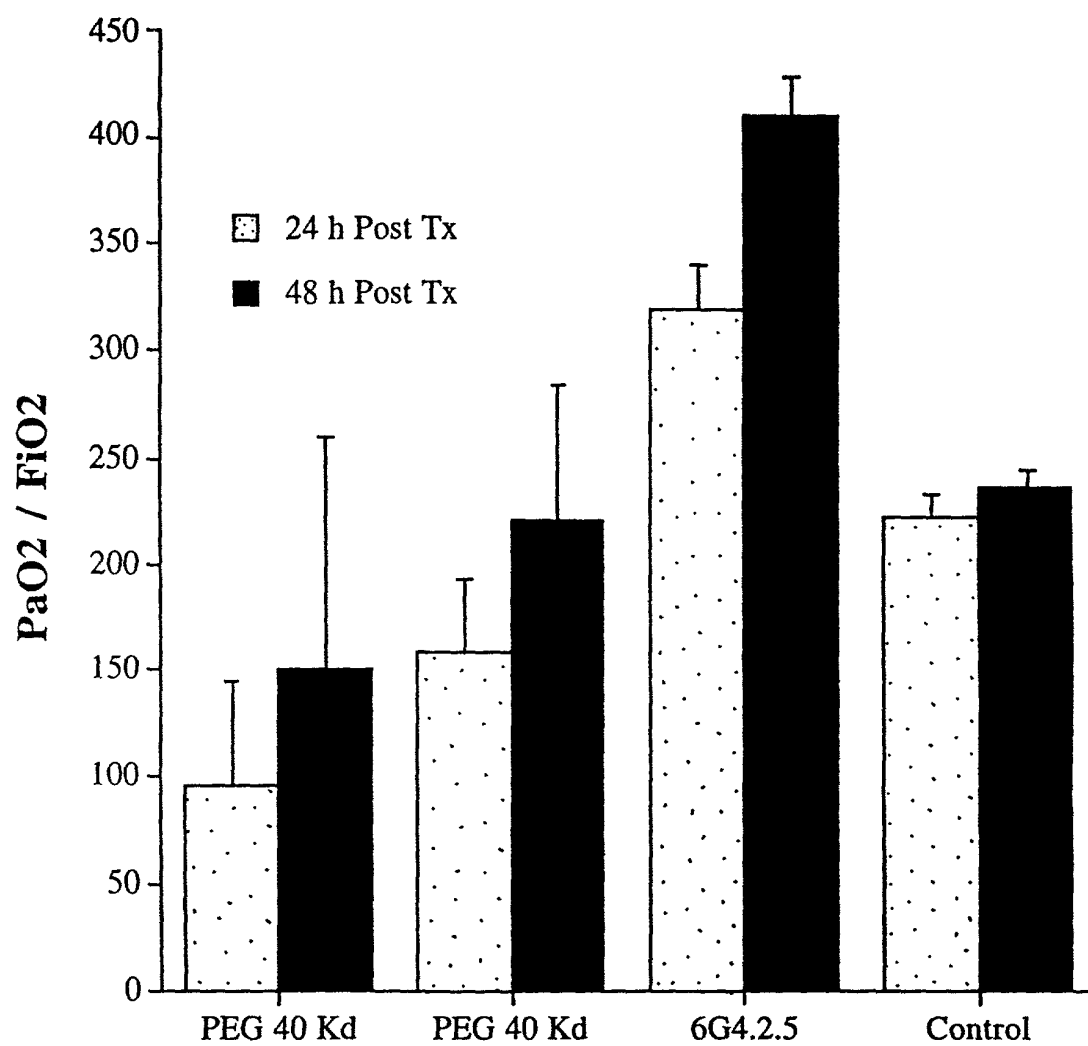


FIG. 69

# Oxygenation in 100% O<sub>2</sub> @24 h Post Anti-IL8 Tx

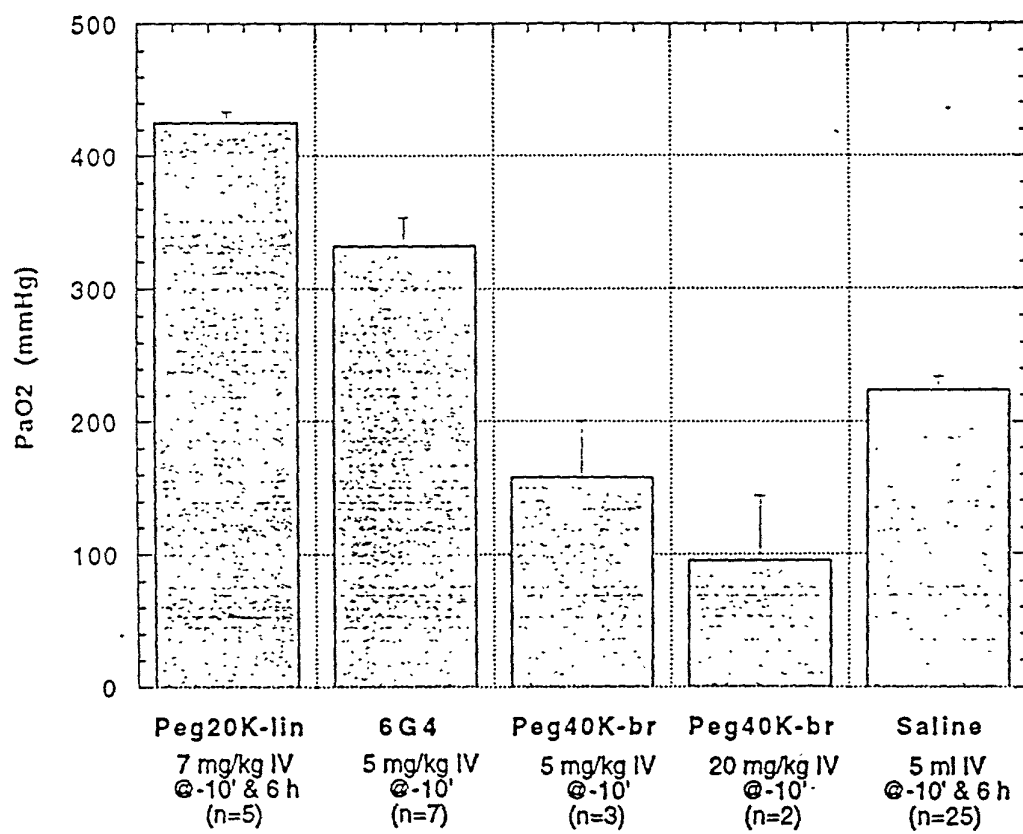


FIG. 70A

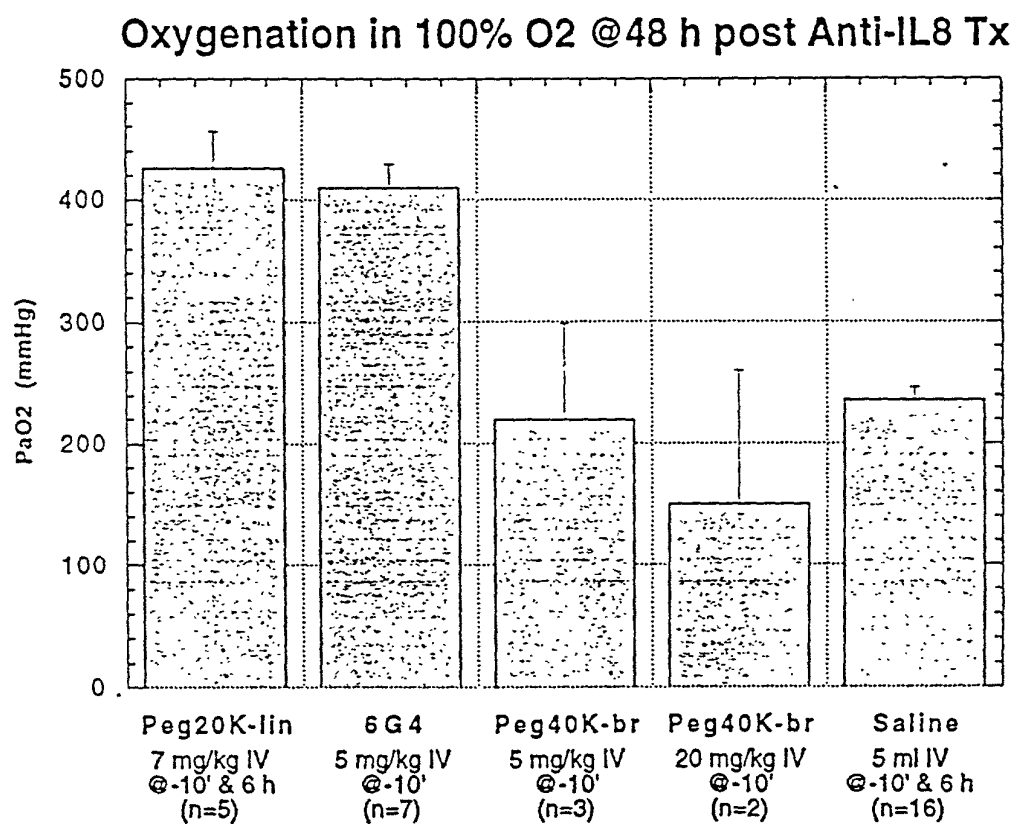


FIG. 70B

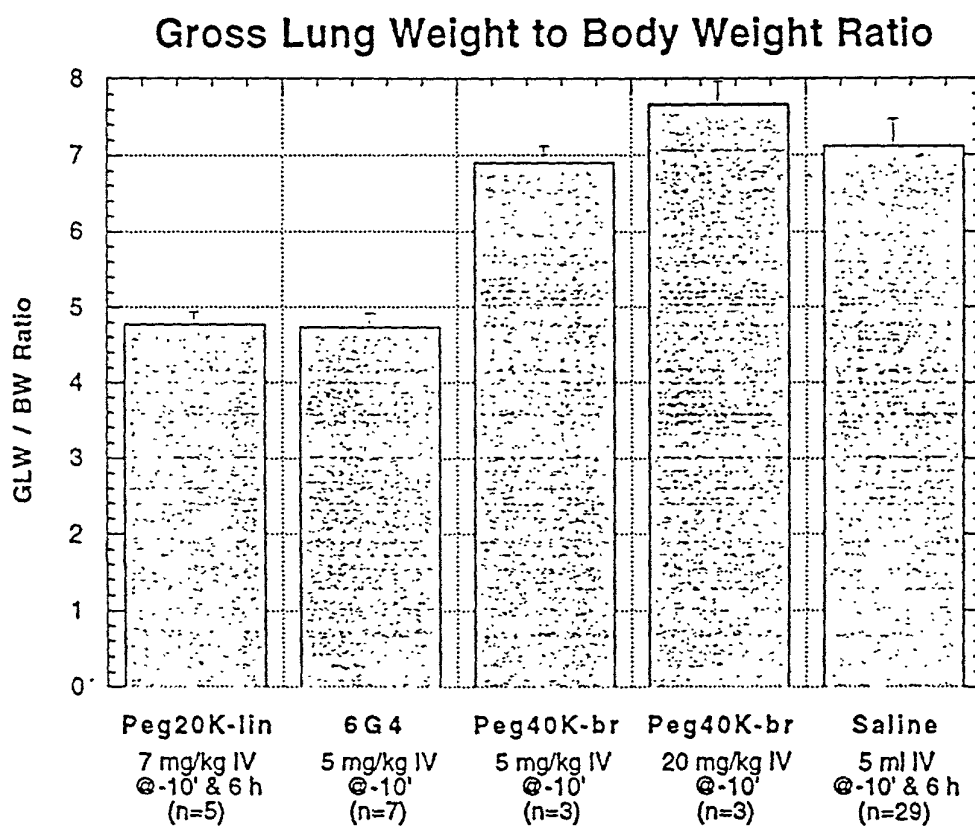


FIG. 70C

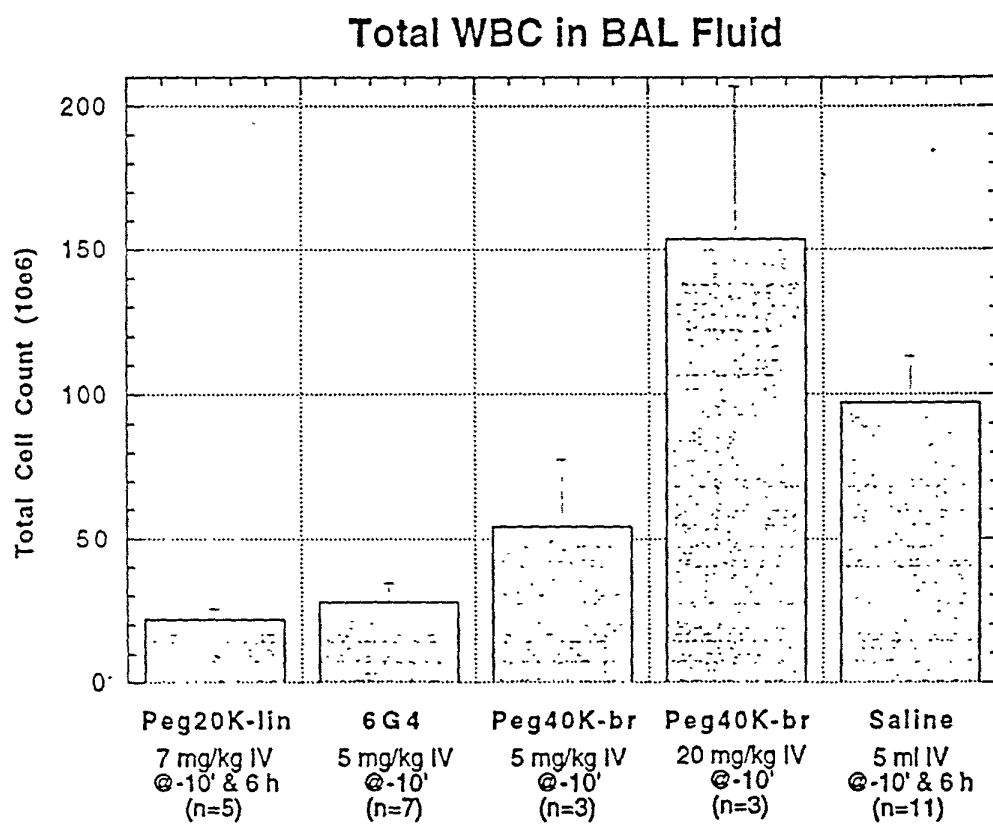


FIG. 70D

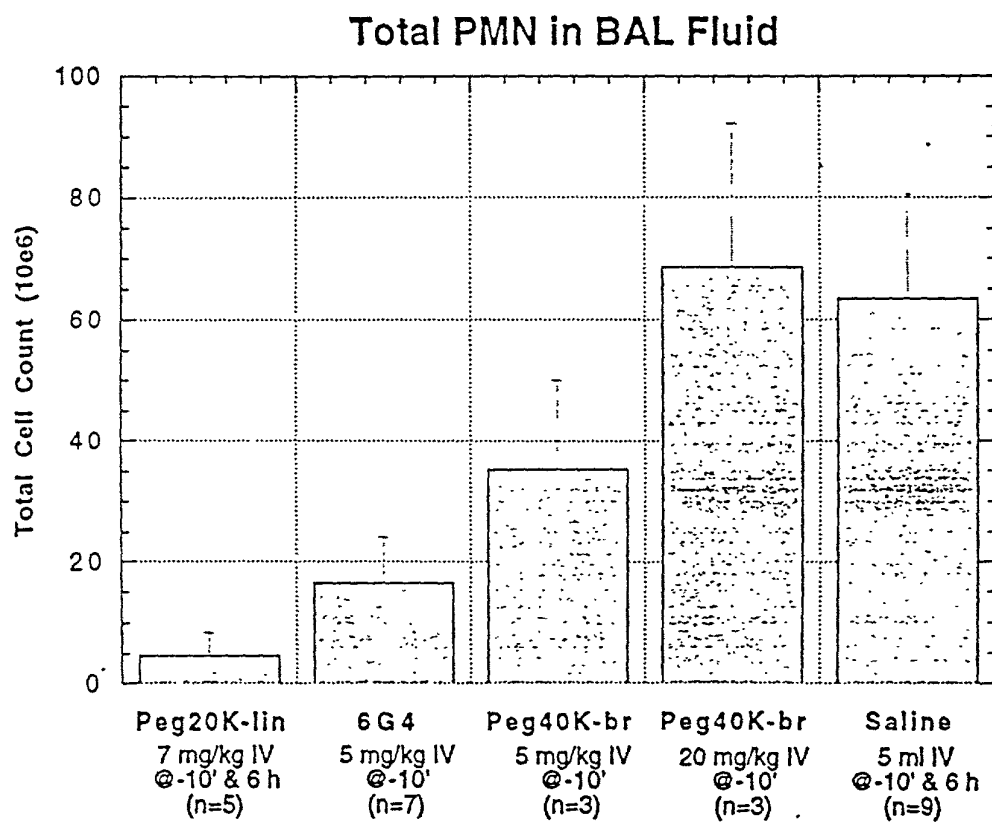
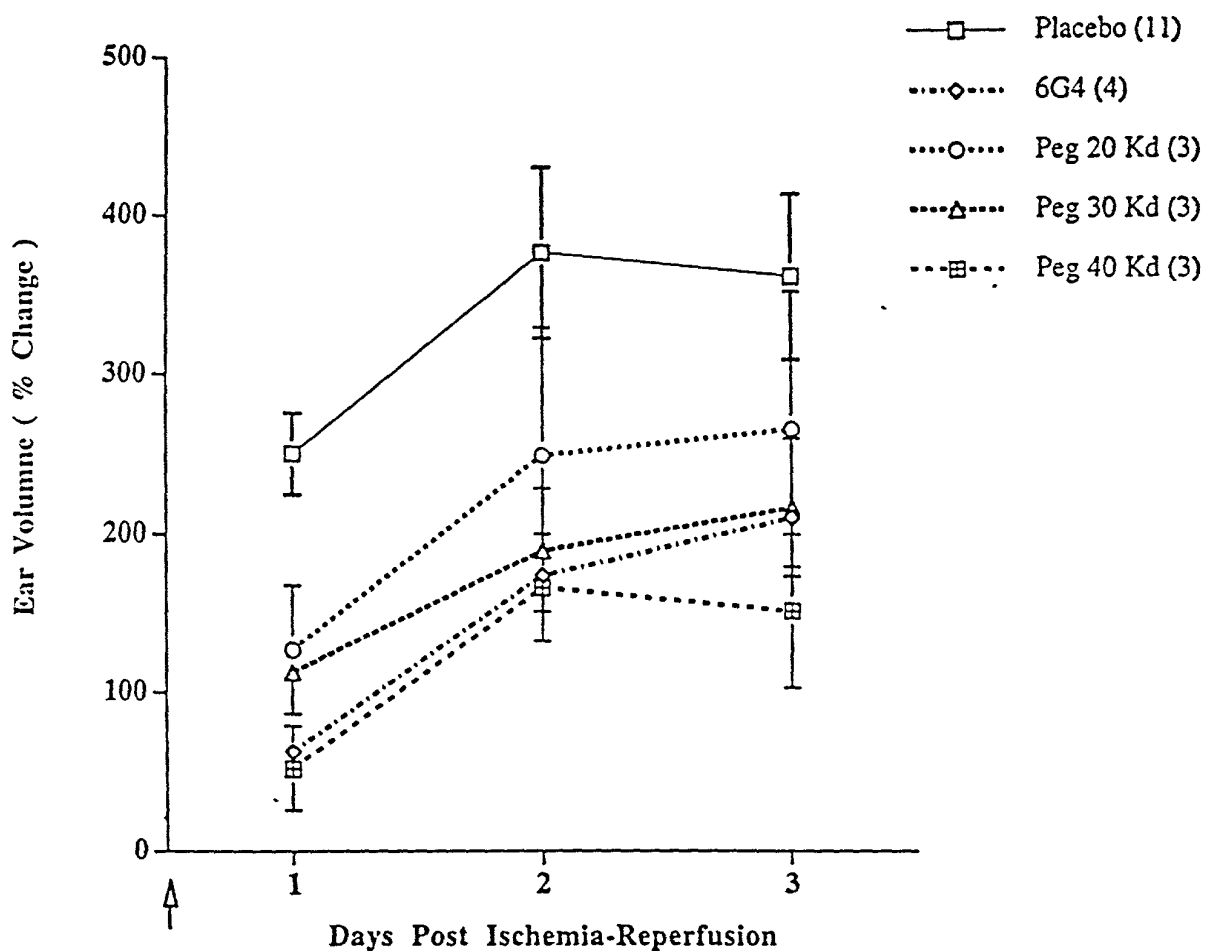


FIG. 70E

## The Effect of Pegylated Anti-IL-8 in the Rabbit Ear model of Ischemia-Reperfusion Injury



Anti-IL-8 formulations :  
Single Dose (5 mg/kg)  
administered IV at time  
of reperfusion

FIG. 71

**Figure 72: 20K and 40K PEG rhuMab VEGF Fab (approximately 3 mg/kg Serum concentrations following IV administration)**

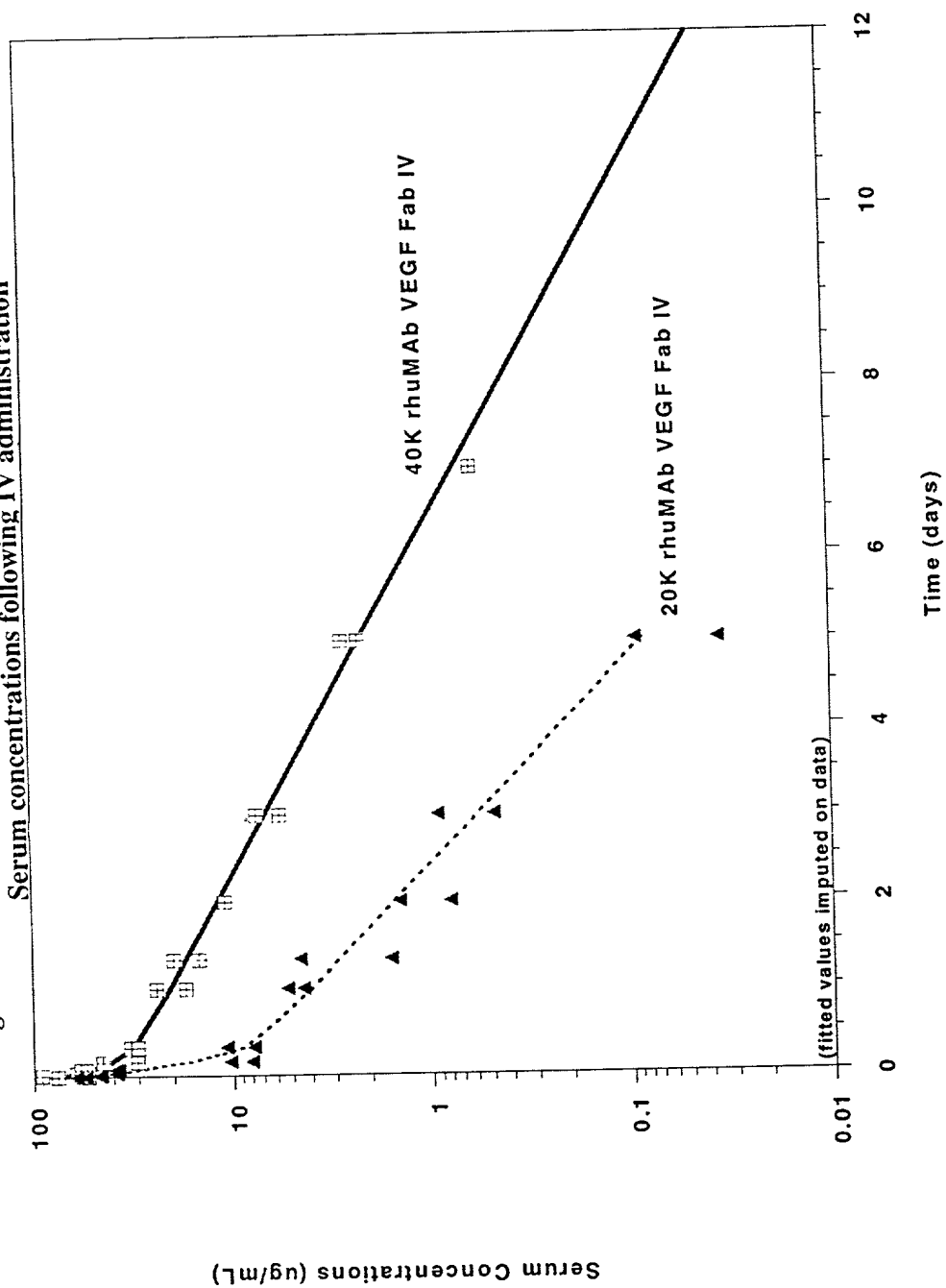
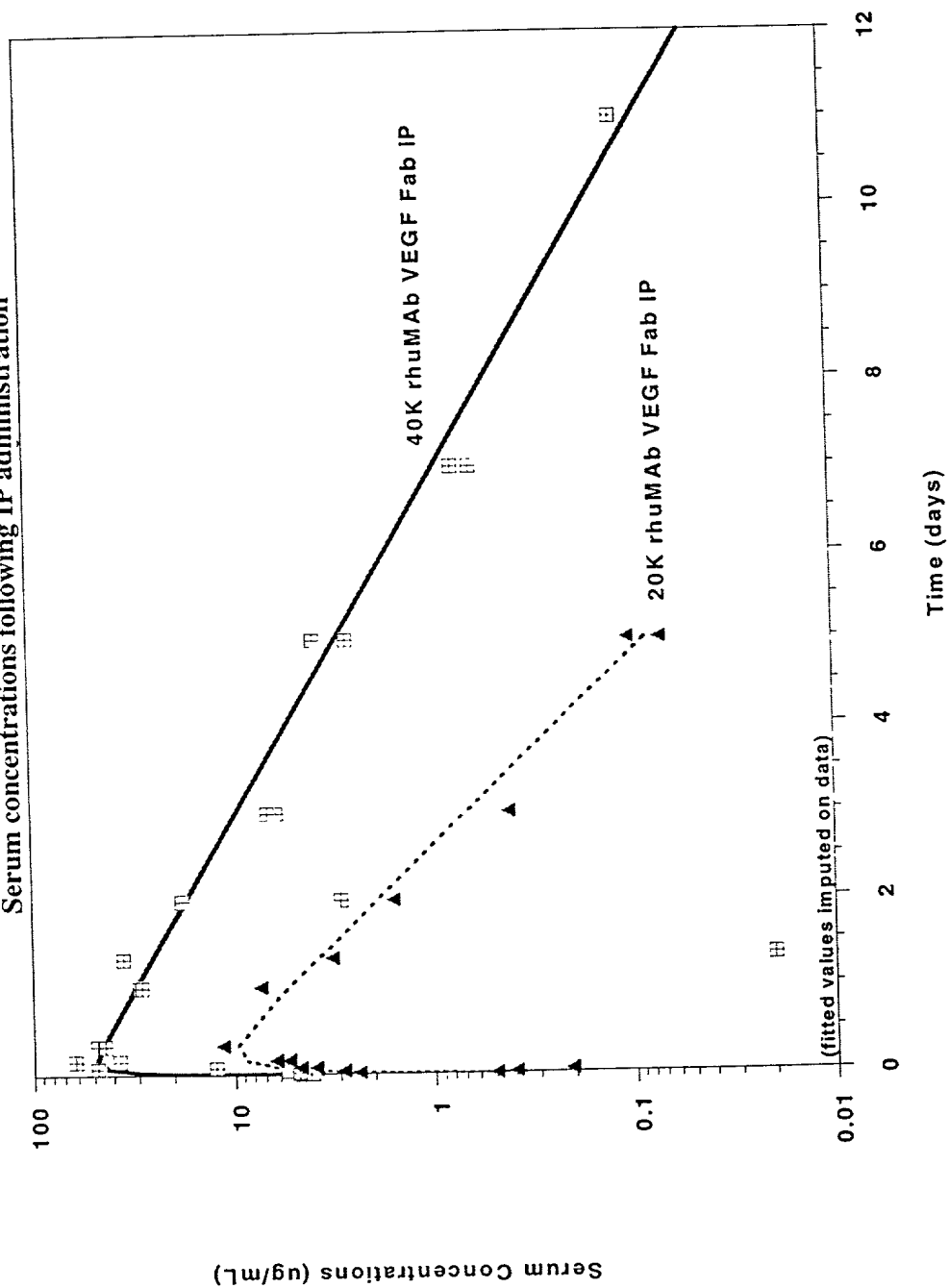




Figure 73: 20K and 40K PEG rhuMab VEGF Fab (approximately 3 mg/kg  
Serum concentrations following IP administration



# TUMOR WEIGHTS AT NECROPSY 98-277

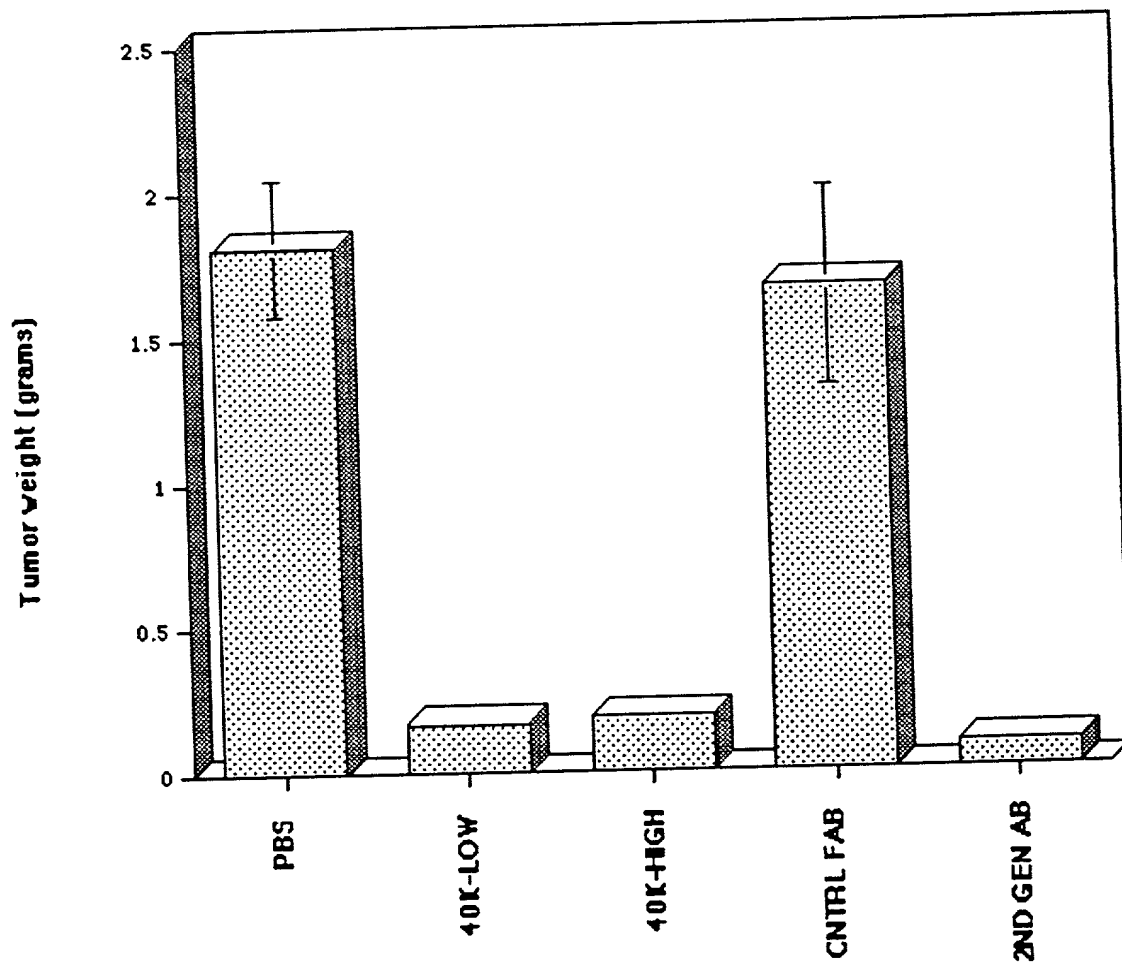


FIG. 74

Sequence Listing

<110> Genentech, Inc., Hsei, Vanessa  
Koumenis, Iphigenia  
Leong, Steven R.  
Shahrokh, Zahra  
Zapata, Gerardo A.

<120> ANTIBODY FRAGMENT-POLYMER CONJUGATES AND USES OF SAME

<130> P1085R6

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<210> 3

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 cctggtatca acagaaacca gggcaatctc ctaaagcact gatttactcg 150  
 tcacctacc ggtacagtgg agtcctgat cgcttcacag gcagtggatc 200  
 tgggacagat ttactctca ccatcagcca tgtgcagtct gaagacttgg 250  
 cagactatct ctgtcagcaa tataacatct atcctctcac gttcggctct 300  
 gggaccaagc tggagttgaa acgggctgat gctgcaccac caactgtatc 350  
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 35 40 45  
 Ala Leu Ile Tyr Ser Ser Ser Tyr Arg Tyr Ser Gly Val Pro Asp  
 50 55 60  
 Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile  
 65 70 75  
 Ser His Val Gln Ser Glu Asp Leu Ala Asp Tyr Phe Cys Gln Gln  
 80 85 90



110

115

120

Ala Lys Thr Thr Ala Pro Ser Val Tyr Pro  
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&lt;213&gt; Artificial Sequence

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&lt;223&gt; recombinant immunoglobulin

&lt;400&gt; 20

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&lt;210&gt; 21

&lt;211&gt; 31

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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&lt;222&gt; 1-31

&lt;223&gt; recombinant immunoglobulin

&lt;400&gt; 21

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&lt;210&gt; 22

&lt;211&gt; 21

&lt;212&gt; DNA

&lt;213&gt; Escherichia coli

&lt;400&gt; 22

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&lt;210&gt; 23

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;221&gt; Artificial Sequence

&lt;222&gt; 1-33

&lt;223&gt; recombinantimmunoglobulin

&lt;400&gt; 23

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&lt;210&gt; 24

&lt;211&gt; 714

&lt;212&gt; DNA

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&lt;221&gt; Artificial Sequence

&lt;222&gt; 1-714

&lt;223&gt; recombinant immunoglobulin

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tgtccacatc agtaggagac agggtcagcg tcacctgcaa ggccagtcag 150  
aatgtgggta ctaatgtagc ctggtatcaa cagaaaccag ggcaatctcc 200  
taaagcactg atttactcgt catcctaccg gtacagtggg gtccctgac 250  
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accctgacgc tgagcaaagc agactacgag aaacacaaaag tctacgcctg 650  
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			20					25						30
Gln	Lys	Phe	Met	Ser	Thr	Ser	Val	Gly	Asp	Arg	Val	Ser	Val	Thr
			35					40						45
Cys	Lys	Ala	Ser	Gln	Asn	Val	Gly	Thr	Asn	Val	Ala	Trp	Tyr	Gln
			50					55						60
Gln	Lys	Pro	Gly	Gln	Ser	Pro	Lys	Ala	Leu	Ile	Tyr	Ser	Ser	Ser
			65					70						75
Tyr	Arg	Tyr	Ser	Gly	Val	Pro	Asp	Arg	Phe	Thr	Gly	Ser	Gly	Ser
			80					85						90



Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser	His	Val	Gln	Ser	Glu	Asp	
				95					100					105	
Leu	Ala	Asp	Tyr	Phe	Cys	Gln	Gln	Tyr	Asn	Ile	Tyr	Pro	Leu	Thr	
				110					115					120	
Phe	Gly	Pro	Gly	Thr	Lys	Leu	Glu	Leu	Arg	Arg	Ala	Val	Ala	Ala	
				125					130					135	
Pro	Ser	Val	Phe	Ile	Phe	Pro	Pro	Ser	Asp	Glu	Gln	Leu	Lys	Ser	
				140					145					150	
Gly	Thr	Ala	Ser	Val	Val	Cys	Leu	Leu	Asn	Asn	Phe	Tyr	Pro	Arg	
				155					160					165	
Glu	Ala	Lys	Val	Gln	Trp	Lys	Val	Asp	Asn	Ala	Leu	Gln	Ser	Gly	
				170					175					180	
Asn	Ser	Gln	Glu	Ser	Val	Thr	Glu	Gln	Asp	Ser	Lys	Asp	Ser	Thr	
				185					190					195	
Tyr	Ser	Leu	Ser	Ser	Thr	Leu	Thr	Leu	Ser	Lys	Ala	Asp	Tyr	Glu	
				200					205					210	
Lys	His	Lys	Val	Tyr	Ala	Cys	Glu	Val	Thr	His	Gln	Gly	Leu	Ser	
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 tagtgccgcc tggagggtcc ctgaaactct cctgtgcagc ctctggattc 150  
 atattcagta gttatggcat gtcttgggtt cgccagactc caggcaagag 200  
 cctggagttg gtcgcaacca ttaataataa tggatgatagc acctattatc 250  
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 acctgtacc tgcaaatgag cagtctgaag tctgaggaca cagccatggt 350  
 ttactgtgca agagccctca ttagttcggc tacttggttt ggttactggg 400  
 gccaaaggac tctggtcact gtctctgcag cctccaccaa gggcccatcg 450  
 gtcttcccc tggcaccctc ctccaagagc acctctgggg gcacagcggc 500



Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser
				200					205					210
Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile
				215					220					225
Cys	Asn	Val	Asn	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys
				230					235					240
Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr				
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 gaaacaccta ttacattgg tacctgcaga agccaggcca gtctccaaag 150  
 ctctgatct acaaagtttc caaccgattt tctgggggtcc cagacaggtt 200  
 cagtggcagt ggatcagga cagatttcac actcaggatc agcagagtgg 250  
 aggetgagga tctgggactt tatttctgct ctcaaagtac acatgttccg 300  
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 35 40 45  
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 Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp  
 65 70 75  
 Phe Thr Leu Arg Ile Ser Arg Val Glu Ala Glu Asp Leu Gly Leu  
 80 85 90  
 Tyr Phe Cys Ser Gln Ser Thr His Val Pro Leu Thr Phe Gly Ala  
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 35 40 45  
 Glu Trp Ile Gly Tyr Ile Asp Pro Ser Asn Gly Glu Thr Thr Tyr  
 50 55 60  
 Asn Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Thr Ser  
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agccttgtag acggtattgg aaacacctat ttacattggt acctgcagaa 200  
gccaggccag tctccaaagc tctgatcta caaagtttcc aaccgatttt 250  
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ctcaggatca gcagagtgga ggctgaggat ctgggacttt atttctgctc 350  
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gagcaattga aatctggaac tgccctgtgt gtgtgcctgc tgaataactt 500  
ctatcccaga gaggccaaag tacagtggaa ggtggataac gccctccaat 550

cgggtaactc ccaggagagt gtcacagagc aggacagcaa ggacagcacc 600  
 tacagcctca gcagcaccct gacgctgagc aaagcagact acgagaaaaca 650  
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<220>  
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 Ser Ile Ala Thr Asn Ala Tyr Ala Asp Ile Val Met Thr Gln Thr  
                     20                    25                    30  
 Pro Leu Ser Leu Pro Val Ser Leu Gly Asp Gln Ala Ser Ile Ser  
                     35                    40                    45  
 Cys Arg Ser Ser Gln Ser Leu Val His Gly Ile Gly Asn Thr Tyr  
                     50                    55                    60  
 Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu  
                     65                    70                    75  
 Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro Asp Arg Phe  
                     80                    85                    90  
 Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Arg Ile Ser Arg  
                     95                    100                    105  
 Val Glu Ala Glu Asp Leu Gly Leu Tyr Phe Cys Ser Gln Ser Thr  
                     110                    115                    120  
 His Val Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys  
                     125                    130                    135  
 Arg Ala Val Ala Ala Pro Thr Val Phe Ile Phe Pro Pro Ser Ser  
                     140                    145                    150  
 Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn  
                     155                    160                    165  
 Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn  
                     170                    175                    180  
 Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp  
                     185                    190                    195  
 Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser  
                     200                    205                    210

CCAGGAGAGT GTCACAGAGC AGGACAGCAA GGACAGCACC 600

Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr  
215 220 225

His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly  
230 235 240

Glu Cys  
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<210> 43

<211> 762

<212> DNA

<213> Artificial Sequence

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<222> 1-762

<223> recombinant immunoglobulin

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tgatgaagcc tggggcttca gtgaagatat cctgcaaggc ttctggttat 150  
tcattcagta gccactacat gcactgggtg aagcagagcc atggaaagag 200  
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ctccagcagc ttgggcaccc agacctacat ctgcaacgtg aatcacaagc 700  
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<210> 44

<211> 253

<212> PRT

<213> Artificial Sequence

<220>

<221> Artificial Sequence

<222> 1-253



<223> recombinant immunoglobulin

<400> 44

Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe  
1 5 10 15

Ser Ile Ala Thr Asn Ala Tyr Ala Glu Ile Gln Leu Gln Gln Ser  
20 25 30

Gly Pro Glu Leu Met Lys Pro Gly Ala Ser Val Lys Ile Ser Cys  
35 40 45

Lys Ala Ser Gly Tyr Ser Phe Ser Ser His Tyr Met His Trp Val  
50 55 60

Lys Gln Ser His Gly Lys Ser Leu Glu Trp Ile Gly Tyr Ile Asp  
65 70 75

Pro Ser Asn Gly Glu Thr Thr Tyr Asn Gln Lys Phe Lys Gly Lys  
80 85 90

Ala Thr Leu Thr Val Asp Thr Ser Ser Ser Thr Ala Asn Val His  
95 100 105

Leu Ser Ser Leu Thr Ser Asp Asp Ser Ala Val Tyr Phe Cys Ala  
110 115 120

Arg Gly Asp Tyr Arg Tyr Asn Gly Asp Trp Phe Phe Asp Val Trp  
125 130 135

Gly Ala Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr Lys Gly  
140 145 150

Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly  
155 160 165

Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu  
170 175 180

Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val  
185 190 195

His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu  
200 205 210

Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr  
215 220 225

Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp  
230 235 240

Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr  
245 250 253

<210> 45

<211> 114

<212> PRT

<213> Mus musculus

<400> 45

Asp Ile Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu

1	5	10	15
Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val	20	25	30
His Gly Ile Gly Asn Thr Tyr Leu His Trp Tyr Leu Gln Lys Pro	35	40	45
Gly Gln Ser Pro Lys Leu Leu Ile Tyr Tyr Lys Val Ser Asn Arg	50	55	60
Phe Ser Gly Val Pro Asp Arg Phe Ser Asp Ser Gly Ser Gly Thr	65	70	75
Asp Phe Thr Leu Arg Ile Ser Arg Val Glu Ala Glu Asp Leu Gly	80	85	90
Leu Tyr Phe Cys Ser Gln Ser Thr His Val Pro Leu Thr Phe Gly	95	100	105
Ala Gly Thr Lys Leu Glu Leu Lys Arg	110	114	

<210> 46  
 <211> 114  
 <212> PRT  
 <213> Artificial Sequence  
  
 <220>  
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 <222> 1-114  
 <223> recombinant immunoglobulin

<400> 46
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15
Gly Asp Arg Val Thr Ile Thr Cys Arg Ser Ser Gln Ser Leu Val
20 25 30
His Gly Ile Gly Asn Thr Tyr Leu His Trp Tyr Gln Gln Lys Pro
35 40 45
Gly Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Lys Val Ser Asn Arg
50 55 60
Phe Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr
65 70 75
Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala
80 85 90
Thr Tyr Tyr Cys Ser Gln Ser Thr His Val Pro Leu Thr Phe Gly
95 100 105
Gln Gly Thr Lys Val Glu Ile Lys Arg
110 114

<210> 47  
 <211> 109  
 <212> PRT

<213> Homo sapiens

<400> 47

Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val
1				5					10					15
Gly	Asp	Arg	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Lys	Thr	Ile	Ser
				20					25					30
Lys	Tyr	Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Lys
				35					40					45
Leu	Leu	Ile	Tyr	Tyr	Ser	Gly	Ser	Thr	Leu	Glu	Ser	Gly	Val	Pro
				50					55					60
Ser	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr
				65					70					75
Ile	Ser	Ser	Leu	Gln	Pro	Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Gln
				80					85					90
Gln	His	Asn	Glu	Tyr	Pro	Leu	Thr	Phe	Gly	Gln	Gly	Thr	Lys	Val
				95					100					105
Glu	Ile	Lys	Arg											
				109										

<210> 48

<211> 117

<212> PRT

<213> Mus Musculus

<400> 48

Glu	Ile	Gln	Leu	Gln	Gln	Ser	Gly	Pro	Glu	Leu	Met	Lys	Pro	Gly
1				5					10					15
Ala	Ser	Val	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Tyr	Ser	Phe	Ser
				20					25					30
Ser	His	Tyr	Met	His	Trp	Val	Lys	Gln	Ser	His	Gly	Lys	Ser	Leu
				35					40					45
Glu	Trp	Ile	Gly	Tyr	Ile	Asp	Pro	Ser	Asn	Gly	Glu	Thr	Thr	Tyr
				50					55					60
Asn	Gln	Lys	Phe	Lys	Gly	Lys	Ala	Thr	Leu	Thr	Val	Asp	Thr	Ser
				65					70					75
Ser	Ser	Thr	Ala	Asn	Val	His	Leu	Ser	Ser	Leu	Thr	Ser	Asp	Asp
				80					85					90
Ser	Ala	Val	Tyr	Phe	Cys	Ala	Ala	Arg	Gly	Asp	Tyr	Arg	Tyr	Asn
				95					100					105
Gly	Asp	Trp	Phe	Phe	Asp	Val	Trp	Gly	Ala	Gly	Thr			
				110					115		117			

<210> 49

<211> 117

<212> PRT

<213> Artificial Sequence

<220>  
 <221> Artificial Sequence  
 <222> 1-117  
 <223> recombinant immunoglobulin

<400> 49  
 Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly  
     1                    5                    10                    15  
 Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Ser  
                     20                    25                    30  
 Ser His Tyr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu  
                     35                    40                    45  
 Glu Trp Val Gly Tyr Ile Asp Pro Ser Asn Gly Glu Thr Thr Tyr  
                     50                    55                    60  
 Asn Gln Lys Phe Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser  
                     65                    70                    75  
 Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp  
                     80                    85                    90  
 Thr Ala Val Tyr Tyr Cys Ala Ala Arg Gly Asp Tyr Arg Tyr Asn  
                     95                    100                    105  
 Gly Asp Trp Phe Phe Asp Val Trp Gly Gln Gly Thr  
                     110                    115                    117

<210> 50  
 <211> 116  
 <212> PRT  
 <213> Homo sapiens

<400> 50  
 Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly  
     1                    5                    10                    15  
 Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Ser Phe Thr  
                     20                    25                    30  
 Gly His Trp Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu  
                     35                    40                    45  
 Glu Trp Val Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Tyr  
                     50                    55                    60  
 Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser  
                     65                    70                    75  
 Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp  
                     80                    85                    90  
 Thr Ala Val Tyr Tyr Cys Ala Ala Arg Gly Ile Tyr Phe Tyr Gly  
                     95                    100                    105  
 Thr Thr Tyr Phe Asp Tyr Trp Gly Gln Gly Thr  
                     110                    115                    116

<210> 51  
<211> 242  
<212> PRT  
<213> Artificial Sequence

<220>  
<221> Artificial Sequence  
<222> 1-242  
<223> recombinant immunoglobulin

<400> 51  
Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe  
1 5 10 15  
Ser Ile Ala Thr Asn Ala Tyr Ala Asp Ile Gln Met Thr Gln Ser  
20 25 30  
Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr  
35 40 45  
Cys Arg Ser Ser Gln Ser Leu Val His Gly Ile Gly Asn Thr Tyr  
50 55 60  
Leu His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu  
65 70 75  
Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro Ser Arg Phe  
80 85 90  
Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser  
95 100 105  
Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Ser Gln Ser Thr  
110 115 120  
His Val Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys  
125 130 135  
Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp  
140 145 150  
Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn  
155 160 165  
Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn  
170 175 180  
Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp  
185 190 195  
Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser  
200 205 210  
Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr  
215 220 225  
His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly  
230 235 240  
Glu Cys  
242

<210> 52  
 <211> 253  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <221> Artificial Sequence  
 <222> 1-253  
 <223> recombinant immunoglobulin

<400> 52  
 Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe  
   1                  5                  10                  15  
 Ser Ile Ala Thr Asn Ala Tyr Ala Glu Val Gln Leu Val Gln Ser  
                   20                  25                  30  
 Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys  
                   35                  40                  45  
 Ala Ala Ser Gly Tyr Ser Phe Ser Ser His Tyr Met His Trp Val  
                   50                  55                  60  
 Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Gly Tyr Ile Asp  
                   65                  70                  75  
 Pro Ser Asn Gly Glu Thr Thr Tyr Asn Gln Lys Phe Lys Gly Arg  
                   80                  85                  90  
 Phe Thr Leu Ser Arg Asp Asn Ser Lys Asn Thr Ala Tyr Leu Gln  
                   95                  100                  105  
 Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala  
                   110                  115                  120  
 Arg Gly Asp Tyr Arg Tyr Asn Gly Asp Trp Phe Phe Asp Val Trp  
                   125                  130                  135  
 Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly  
                   140                  145                  150  
 Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly  
                   155                  160                  165  
 Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu  
                   170                  175                  180  
 Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val  
                   185                  190                  195  
 His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu  
                   200                  205                  210  
 Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr  
                   215                  220                  225  
 Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp  
                   230                  235                  240  
 Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr

245

250

253

<210> 53  
 <211> 159  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <221> Artificial Sequence  
 <222> 1-159  
 <223> recombinant phage protein

<400> 53  
 Ser Gly Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met  
     1                    5                    10                    15  
 Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn  
                     20                    25                    30  
 Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr  
                     35                    40                    45  
 Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly  
                     50                    55                    60  
 Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly Ser Ser  
                     65                    70                    75  
 Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu  
                     80                    85                    90  
 Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val  
                     95                    100                    105  
 Glu Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr Glu Phe  
                     110                    115                    120  
 Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe Ala  
                     125                    130                    135  
 Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser Thr Phe  
                     140                    145                    150  
 Ala Asn Ile Leu Arg Asn Lys Glu Ser  
                     155                    159

<210> 54  
 <211> 780  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> Artificial Sequence  
 <222> 1-780  
 <223> recombinant immunoglobulin

<400> 54  
 atgaaaaaga atatcgcat tcttcttgca tctatgttcg ttttttctat 50  
 tgctacaaac gcatacgctg atatccagat gacccagtcc ccgagctccc 100

tgtccgcctc tgtgggcgat agggtcacca tcacctgcag gtcaagtcaa 150  
 agcttagtac atggtatagg taacacgtat ttactctggg atcaacagaa 200  
 accaggaaaa gctccgaaac tactgattta caaagtatcc aatcgattct 250  
 ctggagtcct ttctcgcttc tctggatccg gttctgggac ggatttcact 300  
 ctgaccatca gcagtctgca gccagaagac ttcgcaactt attactgttc 350  
 acagagtact catgtcccgc tcaogtttgg acagggtacc aaggtggaga 400  
 tcaaacgaac tgtggctgca ccatctgtct tcatcttccc gccatctgat 450  
 gagcagttga aatctggaac tgcttctggt gtgtgcctgc tgaataactt 500  
 ctatcccaga gaggccaaag tacagtggaa ggtggataac gccctccaat 550  
 cgggtaactc ccaggagagt gtcacagagc aggacagcaa ggacagcacc 600  
 tacagcctca gcagcaccct gacgtgagc aaagcagact acgagaaaca 650  
 caaagtctac gcctgcgaag tcacccatca gggcctgagc tcgcccgtca 700  
 caaagagctt caacagggga gagtgtaag ctgacacctc acgccggacg 750  
 catcgtggcc ctagtacgca actagtcgta 780

<210> 55

<211> 253

<212> PRT

<213> Artificial Sequence

<220>

<221> Artificial Sequence

<222> 1-253

<223> recombinant immunoglobulin

<400> 55

Met	Lys	Lys	Asn	Ile	Ala	Phe	Leu	Leu	Ala	Ser	Met	Phe	Val	Phe	1	5	10	15
Ser	Ile	Ala	Thr	Asn	Ala	Tyr	Ala	Glu	Val	Gln	Leu	Val	Glu	Ser	20	25	30	
Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	Gly	Ser	Leu	Arg	Leu	Ser	Cys	35	40	45	
Ala	Ala	Ser	Gly	Tyr	Ser	Phe	Ser	Ser	His	Tyr	Met	His	Trp	Val	50	55	60	
Lys	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Val	Gly	Tyr	Ile	Asp	65	70	75	
Pro	Ser	Asn	Gly	Glu	Thr	Thr	Tyr	Asn	Gln	Lys	Phe	Lys	Gly	Arg	80	85	90	
Phe	Thr	Leu	Ser	Arg	Asp	Asn	Ser	Lys	Asn	Thr	Ala	Tyr	Leu	Gln	95	100	105	
Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys	Ala				



	110	115	120
Arg Gly Asp Tyr	Arg Tyr Asn Gly Asp	Trp Phe Phe Asp Val	Trp
	125	130	135
Gly Gln Gly Thr	Leu Val Thr Val Ser	Ser Ala Ser Thr Lys	Gly
	140	145	150
Pro Ser Val Phe	Pro Leu Ala Pro Ser	Ser Lys Ser Thr Ser	Gly
	155	160	165
Gly Thr Ala Ala	Leu Gly Cys Leu Val	Lys Asp Tyr Phe Pro	Glu
	170	175	180
Pro Val Thr Val	Ser Trp Asn Ser Gly	Ala Leu Thr Ser Gly	Val
	185	190	195
His Thr Phe Pro	Ala Val Leu Gln Ser	Ser Gly Leu Tyr Ser	Leu
	200	205	210
Ser Ser Val Val	Thr Val Pro Ser Ser	Ser Leu Gly Thr Gln	Thr
	215	220	225
Tyr Ile Cys Asn	Val Asn His Lys Pro	Ser Asn Thr Lys Val	Asp
	230	235	240
Lys Lys Val Glu	Pro Lys Ser Cys Asp	Lys Thr His Thr	
	245	250	253

<210> 56  
 <211> 242  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <221> Artificial Sequence  
 <222> 1-242  
 <223> recombinant immunoglobulin

<400> 56  
 Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe  
 1 5 10 15  
 Ser Ile Ala Thr Asn Ala Tyr Ala Asp Ile Gln Met Thr Gln Ser  
 20 25 30  
 Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr  
 35 40 45  
 Cys Arg Ser Ser Gln Ser Leu Val His Gly Ile Gly Ala Thr Tyr  
 50 55 60  
 Leu His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu  
 65 70 75  
 Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro Ser Arg Phe  
 80 85 90  
 Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser  
 95 100 105

Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Ser Gln Ser Thr  
110 115 120

His Val Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys  
125 130 135

Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp  
140 145 150

Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn  
155 160 165

Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn  
170 175 180

Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp  
185 190 195

Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser  
200 205 210

Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr  
215 220 225

His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly  
230 235 240

Glu Cys  
242

<210> 57

<211> 45

<212> PRT

<213> Artificial Sequence

<220>

<221> Artificial Sequence

<222> 1-45

<223> recombinant leucine zipper peptide

<400> 57

Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Arg Met Lys  
1 5 10 15

Gln Leu Glu Asp Lys Val Glu Glu Leu Leu Ser Lys Asn Tyr His  
20 25 30

Leu Glu Asn Glu Val Ala Arg Leu Lys Lys Leu Val Gly Glu Arg  
35 40 45

<210> 58

<211> 780

<212> DNA

<213> Artificial Sequence

<220>

<221> Artificial Sequence

<222> 1-780

<223> recombinant immunoglobulin

<400> 58

atgaaaaaga atatcgcat tcttcttgca tctatgttcg ttttttctat 50  
 tgctacaaac gcatacgctg atatccagat gaccagtc ccgagctccc 100  
 tgtccgcctc tgtgggcgat agggtcacca tcacctgcag gtcaagtcaa 150  
 agcttagtac atggtatagg tgctacgtat ttacactgg atcaacagaa 200  
 accaggaaaa gctccgaaac tactgattta caaagtatcc aatcgattct 250  
 ctggagtccc ttctcgcttc tctggatccg gttctgggac ggatttcact 300  
 ctgaccatca gcagtctgca gccagaagac ttcgcaactt attactgttc 350  
 acagagtact catgtcccg ctcacgtttgg acagggtagc aaggtggaga 400  
 tcaaacgaac tgtggctgca ccatctgtct tcattctccc gccatctgat 450  
 gagcagttga aatctggaac tgcttctgtt gtgtgactgc tgaataactt 500  
 ctatcccaga gaggccaaag tacagtggaa ggtggataac gccctccaat 550  
 cgggtaactc ccaggagagt gtcacagagc aggacagcaa ggacagcacc 600  
 tacagcctca gcagcacct gacgtgagc aaagcagact acgagaaaca 650  
 caaagtctac gcctgcgaag tcacccatca gggcctgagc tcgcccgtca 700  
 caaagagctt caacagggga gagtgtaaag ctgacctct acgccggacg 750  
 catcgtggcc ctagtacgca actagtcgta 780

<210> 59

<211> 927

<212> DNA

<213> Artificial Sequence

<220>

<221> Artificial Sequence

<222> 1-927

<223> recombinant immunoglobulin

<400> 59

aaaagggtat ctagagggtg aggtgatttt atgaaaaaga atatcgcat 50  
 tcttcttgca tctatgttcg ttttttctat tgctacaaac gcgtacgctg 100  
 aggttcagct agtgcagtct ggcgggtggc tgggtgcagcc agggggctca 150  
 ctccgtttgt cctgtgcagc ttctggctac tccttctcga gtcactatat 200  
 gcactgggtc cgtcaggccc cgggtaaggg cctggaatgg gttggatata 250  
 ttgaccttc caatgggtgaa actacgtata atcaaaagtt caagggccgt 300  
 ttacttttat ctgcgcgaaa ctccaaaaac acagcatacc tgcagatgaa 350  
 cagcctgcgt gctgaggaca ctgccgtcta ttactgtgca agaggggatt 400  
 atcgctacaa tggtgactgg ttcttcgacg tctgggggtca aggaaccctg 450

gtcacctgtct cctcggcctc caccaagggc ccacgtgtct tccccctggc 500  
accctcctcc aagagcacct ctggggggcac agcggccctg ggctgcctgg 550  
tcaaggacta cttccccgaa ccggtgacgg tgtcgtggaa ctcaggcgcc 600  
ctgaccagcg gcgtgcacac cttcccggtt gtcctacagt cctcaggact 650  
ctactccctc agcagcgtgg tgaccgtgcc ctccagcagc ttgggcaccc 700  
agacctacat ctgcaacgtg aatcacaagc ccagcaacac caaggtcgac 750  
aagaaagttg agcccaaata ttgtgacaaa actcacacat gcccgccgtg 800  
cccagcacca gaactgctgg gcggccgcgt gaaacagcta gaggacaagg 850  
tcgaagagct actctccaag aactaccacc tagagaatga agtggcaaga 900  
ctcaaaaagc ttgtcgggga gcgctaa 927

<210> 60

<211> 298

<212> PRT

<213> Artificial Sequence

<220>

<221> Artificial Sequence

<222> 1-298

<223> recombinant immunoglobulin

<400> 60

Met	Lys	Lys	Asn	Ile	Ala	Phe	Leu	Leu	Ala	Ser	Met	Phe	Val	Phe	1	5	10	15
Ser	Ile	Ala	Thr	Asn	Ala	Tyr	Ala	Glu	Val	Gln	Leu	Val	Gln	Ser	20	25	30	
Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	Gly	Ser	Leu	Arg	Leu	Ser	Cys	35	40	45	
Ala	Ala	Ser	Gly	Tyr	Ser	Phe	Ser	Ser	His	Tyr	Met	His	Trp	Val	50	55	60	
Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Val	Gly	Tyr	Ile	Asp	65	70	75	
Pro	Ser	Asn	Gly	Glu	Thr	Thr	Tyr	Asn	Gln	Lys	Phe	Lys	Gly	Arg	80	85	90	
Phe	Thr	Leu	Ser	Arg	Asp	Asn	Ser	Lys	Asn	Thr	Ala	Tyr	Leu	Gln	95	100	105	
Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys	Ala	110	115	120	
Arg	Gly	Asp	Tyr	Arg	Tyr	Asn	Gly	Asp	Trp	Phe	Phe	Asp	Val	Trp	125	130	135	
Gly	Gln	Gly	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	140	145	150	

Pro	Ser	Val	Phe	Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	
				155					160					165	
Gly	Thr	Ala	Ala	Leu	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	
				170					175					180	
Pro	Val	Thr	Val	Ser	Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	
				185					190					195	
His	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	
				200					205					210	
Ser	Ser	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	
				215					220					225	
Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	
				230					235					240	
Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	
				245					250					255	
Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Arg	Met	Lys	Gln	Leu	
				260					265					270	
Glu	Asp	Lys	Val	Glu	Glu	Leu	Leu	Ser	Lys	Asn	Tyr	His	Leu	Glu	
				275					280					285	
Asn	Glu	Val	Ala	Arg	Leu	Lys	Lys	Leu	Val	Gly	Glu	Arg			
				290					295			298			

<210> 61  
 <211> 6563  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> Artificial Sequence  
 <222> 1-6563  
 <223> recombinant immunoglobulin

<400> 61  
 gaattcaact tctccatact ttggataagg aaatacagac atgaaaaatc 50  
 tcatttgctga gttgttattt aagcttgccc aaaaagaaga agagtcgaat 100  
 gaactgtgtg cgcaggtaga agctttggag attatcgtca ctgcaatgct 150  
 tcgcaatatg gcgcaaaatg accaacagcg gttgattgat caggtagagg 200  
 gggcgctgta cgaggtaaag cccgatgccg gcattcctga cgacgatacg 250  
 gagctgctgc gcgattacgt aaagaagtta ttgaagcatc ctcgtcagta 300  
 aaaagttaat cttttcaaca gctgtcataa agttgtcacg gccgagactt 350  
 atagtcgctt tgtttttatt ttttaatgta tttgtaacta gaattcgagc 400  
 tcggtacccg gggatcctct cgagggttgag gtgattttat gaaaaagaat 450  
 atcgcatctt ttcttgcatc tatgttcggt ttttctattg ctacaaacgc 500

atacgctgat atccagatga cccagtcgcc gagctccctg tccgcctctg 550  
 tgggcgatag ggtcaccatc acctgcaggt caagtcaaag cttagtacat 600  
 ggtataggtg ctacgtatct acactgggtat caacagaaac caggaaaagc 650  
 tccgaaacta ctgattttaca aagtatccaa tcgattctct ggagtccttt 700  
 ctgcctttct tggatccggt tctgggacgg atttcaactct gaccatcagc 750  
 agtctgcagc cagaagactt cgcaacttat tactgtttac agagtactca 800  
 tgtcccgctc acgtttggac agggtagcaa ggtggagatc aaacgaactg 850  
 tggctgcacc atctgtcttc atcttcccg cactctgatga gcagttgaaa 900  
 tctggaactg cttctgttgt gtgcctgctg aataacttct atcccagaga 950  
 ggccaaagta cagtgggaag tggataacgc cctccaatcg ggtaactccc 1000  
 aggagagtgt cacagagcag gacagcaagg acagcaccta cagcctcagc 1050  
 agcacctga cgctgagcaa agcagactac gagaaacaca aagtctacgc 1100  
 ctgcgaagtc acccatcagg gcttgagctc gcccgtcaca aagagcttca 1150  
 acaggggaga gtgttaagct gatcctctac gccggacgca tcgtggccct 1200  
 agtacgcaac tagtcgtaaa aagggtatct agaggttgag gtgattttat 1250  
 gaaaaagaat atcgcatctt ttcttgcatc tatgttcggt tttctattg 1300  
 ctacaaacgc gtacgctgag gttcagctag tgcagtctgg cggtagcctg 1350  
 gtgcagccag ggggctcact ccgtttgtcc tgtgcagctt ctggctactc 1400  
 cttctcgagt cactatatgc actgggtccg tcaggccccc ggtaaggggc 1450  
 tggaatgggt tggatatatt gatccttcca atggtgaaac tacgtataat 1500  
 caaaagttca agggccgttt cactttatct cgcgacaact caaaaaacac 1550  
 agcatacctg cagatgaaca gcctgcgtgc tgaggacact gccgtctatt 1600  
 actgtgcaag aggggattat cgctacaatg gtgactggtt cttcgacgtc 1650  
 tggggtcaag gaacctgggt caccgtctcc tcggcctcca ccaaggggcc 1700  
 atcggctctc cccctggcac cctcctcaa gagcacctct gggggcacag 1750  
 cggccctggg ctgcctggtc aaggactact tcccgaacc ggtgacggtg 1800  
 tcgtggaact caggcgccct gaccagcggc gtgcacacct tcccggctgt 1850  
 cctacagtcc tcaggactct actccctcag cagcgtggtg accgtgccct 1900  
 ccagcagctt gggcaccag acctacatct gcaacgtgaa tcacaagccc 1950  
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<211> 800

<212> DNA

<213> Artificial Sequence

<220>

<221> Artificial Sequence

<222> 1-800

<223> recombinant immunoglobulin

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<211> 256

<212> PRT

<213> Artificial Sequence

<220>

<221> Artificial Sequence

<222> 1-256

<223> recombinant immunoglobulin

<400> 70

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Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys  
35 40 45

Ala Ala Ser Gly Tyr Ser Phe Ser Ser His Tyr Met His Trp Val  
50 55 60

Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Gly Tyr Ile Asp  
65 70 75

Pro Ser Asn Gly Glu Thr Thr Tyr Asn Gln Lys Phe Lys Gly Arg  
80 85 90

Phe Thr Leu Ser Arg Asp Asn Ser Lys Asn Thr Ala Tyr Leu Gln  
95 100 105

Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala  
110 115 120

Arg Gly Asp Tyr Arg Tyr Asn Gly Asp Trp Phe Phe Asp Val Trp  
125 130 135

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly  
140 145 150

Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly  
155 160 165

Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu  
170 175 180

Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val  
185 190 195

His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu  
200 205 210

Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr  
215 220 225

Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp  
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Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro  
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Pro  
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<210> 71  
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 <212> PRT  
 <213> Artificial Sequence

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 <222> 1-452  
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                   20                  25                  30  
 Ser His Tyr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu  
                   35                  40                  45  
 Glu Trp Val Gly Tyr Ile Asp Pro Ser Asn Gly Glu Thr Thr Tyr  
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 Asn Gln Lys Phe Lys Gly Arg Phe Thr Leu Ser Arg Asp Asn Ser  
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 Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp  
                   80                  85                  90  
 Thr Ala Val Tyr Tyr Cys Ala Arg Gly Asp Tyr Arg Tyr Asn Gly  
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 Asp Trp Phe Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val  
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                   215                  220                  225  
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 Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr

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Leu Met Ile Ser	Arg Thr Pro Glu Val	Thr Cys Val Val Val	Asp		
	260	265	270		
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	275	280	285		
Gly Val Glu Val	His Asn Ala Lys Thr	Lys Pro Arg Glu Glu	Gln		
	290	295	300		
Tyr Asn Ser Thr	Tyr Arg Val Val Ser	Val Leu Thr Val Leu	His		
	305	310	315		
Gln Asp Trp Leu	Asn Gly Lys Glu Tyr	Lys Cys Lys Val Ser	Asn		
	320	325	330		
Lys Ala Leu Pro	Ala Pro Ile Glu Lys	Thr Ile Ser Lys Ala	Lys		
	335	340	345		
Gly Gln Pro Arg	Glu Pro Gln Val Tyr	Thr Leu Pro Pro Ser	Arg		
	350	355	360		
Glu Glu Met Thr	Lys Asn Gln Val Ser	Leu Thr Cys Leu Val	Lys		
	365	370	375		
Gly Phe Tyr Pro	Ser Asp Ile Ala Val	Glu Trp Glu Ser Asn	Gly		
	380	385	390		
Gln Pro Glu Asn	Asn Tyr Lys Thr Thr	Pro Pro Val Leu Asp	Ser		
	395	400	405		
Asp Gly Ser Phe	Phe Leu Tyr Ser Lys	Leu Thr Val Asp Lys	Ser		
	410	415	420		
Arg Trp Gln Gln	Gly Asn Val Phe Ser	Cys Ser Val Met His	Glu		
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Ala Leu His Asn	His Tyr Thr Gln Lys	Ser Leu Ser Leu Ser	Pro		
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Gly Lys					
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<211> 219

<212> PRT

<213> Artificial Sequence

<220>

<221> Artificial Sequence

<222> 1-219

<223> recombinant imunoglobulin

<400> 72

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				20				25					30	

His	Gly	Ile	Gly	Ala	Thr	Tyr	Leu	His	Trp	Tyr	Gln	Gln	Lys	Pro	35	40	45
Gly	Lys	Ala	Pro	Lys	Leu	Leu	Ile	Tyr	Lys	Val	Ser	Asn	Arg	Phe	50	55	60
Ser	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	65	70	75
Phe	Thr	Leu	Thr	Ile	Ser	Ser	Leu	Gln	Pro	Glu	Asp	Phe	Ala	Thr	80	85	90
Tyr	Tyr	Cys	Ser	Gln	Ser	Thr	His	Val	Pro	Leu	Thr	Phe	Gly	Gln	95	100	105
Gly	Thr	Lys	Val	Glu	Ile	Lys	Arg	Thr	Val	Ala	Ala	Pro	Ser	Val	110	115	120
Phe	Ile	Phe	Pro	Pro	Ser	Asp	Glu	Gln	Leu	Lys	Ser	Gly	Thr	Ala	125	130	135
Ser	Val	Val	Cys	Leu	Leu	Asn	Asn	Phe	Tyr	Pro	Arg	Glu	Ala	Lys	140	145	150
Val	Gln	Trp	Lys	Val	Asp	Asn	Ala	Leu	Gln	Ser	Gly	Asn	Ser	Gln	155	160	165
Glu	Ser	Val	Thr	Glu	Gln	Asp	Ser	Lys	Asp	Ser	Thr	Tyr	Ser	Leu	170	175	180
Ser	Ser	Thr	Leu	Thr	Leu	Ser	Lys	Ala	Asp	Tyr	Glu	Lys	His	Lys	185	190	195
Val	Tyr	Ala	Cys	Glu	Val	Thr	His	Gln	Gly	Leu	Ser	Ser	Pro	Val	200	205	210
Thr	Lys	Ser	Phe	Asn	Arg	Gly	Glu	Cys							215	219	

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